In vitro transdifferentiation of liver into functional pancreatic-like cells

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Abstract

The human HepG2 (hepatoma) cells can be induced to convert into pancreatic cells following introduction of an activated form of the pancreatic transcription factor Pdx1 (Xlhbox8Vp16). In this study, further investigations were carried out to elucidate the cellular and molecular basis of this conversion and to determine whether the transdifferentiated β-cells are functional. The results showed that (1) both pancreatic exocrine cells and endocrine cells are induced; (2) the hepatic phenotype is suppressed and (3) requirement for the transgene is transient during transdifferentiation of liver cells into pancreatic-like cells. The transdifferentiated cells are functional based on (1) expression of the β-cell functional markers prohormone convertase 1/3 and C-peptide; (2) insulin mRNA expression is increased after treatment with GLP-1 and betacellulin; and (3) insulin secretion is elevated after a glucose challenge.

To verify whether transdifferentiation can also occur in primary cultures of hepatocytes, an *in vitro* culture system for differentiated rat hepatocytes was established using a chemically-defined medium (Keratinocyte serum free medium supplemented with dexamethasone, epidermal growth factor and pituitary gland extract (KDS)). KDS was able to maintain primary rat hepatocytes for up to 28 days in culture without significant loss of liver-specific properties based on the maintenance of morphological appearance, expression of liver proteins and liver-enriched transcription factors and certain hepatic functions including glycogen storage, ureagenesis and xenobiotic metabolism. Cultured rat hepatocytes can be also converted into insulin-producing cells by introducing the transgene Xlhbox8Vp16. A more mature β-cell phenotype (increased expression of β-specific genes (e.g. sulphonylurea receptor and potassium channel Kir6.2)) was seen when additional pancreatic transcription factors (Neurogenin 3, Nkx2.2 and Nkx6.1) were introduced in the cells. These studies suggested the hepatocytes are a potential source of β-cell for therapeutic therapy into diabetics.

Abbreviations

AFP	α1-fetoprotein
AGEs	Advanced glycosylation end-products
ALEs	Advanced lipoxidation end-products
AMCA	Aminomethylcoumarin acetate
aPKC	atypical protein kinase C
ApoB	Apolipoprotein B
APTS	3-aminopropyltriethoxysilane
bHLH	basic helix-loop-helix
BMDC	Bone marrow derived cells
BME	Basal Essential Medium
BMP	Bone morphogenetic protein
BPE	Bovine pituitary gland extract
BTC	Betacellulin
cAMP	cyclic 5'-adenosine monophosphate
CAR	Coxsackievirus and adenovirus receptor (Chapter 5)
CAR	Constitutive active receptor (Chapter 6)
cDNA	complementary DNA
C/EBP	CCAAT/enhancer-binding protein
ChA	Chromogranin A
CK	Cytokeratin
CMV	Cytomegalovirus
CPS	Carbamoyl phosphate synthetase
CSII	Continuous subcutaneous insulin infusion
Cx	Connexin

CYPs	Cytochrome P450 proteins
DAPI	4,6-diamidino-2-phenylindole
DDP-IV	Dipepidyl peptidase IV
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylene-diaminetetraacetic acid
rhEGF	(recombinant human) Epidermal growth factor
EGFP	Enhanced green fluorescence protein
EGTA	Ethylene-glycotetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ES cells	Embryonic stem cells
FACS	Fluorescence-activated cell sorting
FAH	Fumaryl-acetoacetate hydrolase
FBS	Foetal Bovine Serum
Fgf	Fibroblast growth factors
FITC	Fluorescein isothiocyanate
Fox	Forkhead box
G-6-P	Glucose-6-phosphate
GAD	Glutamic acid decarboxylase
GCK	Glucokinase
GFP	Green fluorescence protein
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide 1
GLUT	Glucose transporter

GMPs	Granulocyte macrophage progenitors
GS	Glutamine synthetase
GSIR	Glucose-stimulated insulin release
HDAD	Helper-dependent adenovirus
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
Hes1	Hairy/Enhancer of Split-related protein 1
Hex	Haematopoietically expressed homeobox
HGF	Hepatoctye growth factor
HNF	Hepatocyte nuclear factor
Hp	Haptoglobin
HSCs	Hematopoietic stem cells
IAPP	Islet amyloid polypeptide
ICM	Inner cell mass
IL	Interleukin
IP_3	Inositol-1,4,5-triphosphate
IR	Insulin receptor
IRS	Insulin receptor substrate
ISNP	Isonitropropiophenone
LB	Luria bertani
LIP	Liver inhibitory protein
LPL	Lipoprotein lipase
K_{ATP}	ATP-sensitive potassium channel
KGF	Keratinocyte growth factor
KSFM	Keratinocyte serum free medium
Kv	Voltage-gated K^+-channels
MAPK	Mitogen-Activated Protein Kinase

MEM	Minimal Essential Medium
MEMFA	MOPS, EGTA, $MgSO_4$, Fomalin
MG	α2-macroglobulin
MHC	Myosin heavy chain
MLC	Myosin light chain
MODY	Maturity-onset diabetes of the young
MOI	Multiplicity of infection
MOPS	3-N-Morpholino propanesulfonic acid
MRFs	Myogenic regulatory transcription factors
mRNA	messenger RNA
MRP	Multidrug resistant protein
MSCs	Mesenchymal stem cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NBTC	2-(2-nitro-4-trifluoro-methylbenzyol)-1,3-cyclohexanedione
NeuroD1	NeuroD1 / β cell E-box trans-activator 2
NF	Nuclear factor
nGFP	nuclear Green fluorescence protein
Ngn3	Neurogenin 3
Nkx	NK class homeodomain protein
NOD	Non-obese diabetic
PAS	Periodic Acid Schiff
Pax	Paired-box homeobox gene
PC	Prohormone convertase
PDI	Protein-disulfide isomerase
Pdx1	Pancreatic duodenal homeobox 1
PEPCK	Phosphoenolpyruvate carboxykinase

PFA	Paraformaldehyde
PI3K	Phosphatidylinositol-3 kinase
PIP_2	Phosphatidylinositol-4,5-biphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PP	Pancreatic polypeptide
PPAR	Peroxisome proliferating activating receptor
Prox-1	Prospero-related homeobox 1
PS	Primitive streak
PTF1	Pancreatic transcription factor 1
Px	Pancreatectomy
RER	Rough endoplasmic reticulum
RGD	Arginine-glycine-aspartic acid
ROS	Reactive oxygen species
RPE	Retinal pigmented epithelium
RR	Ribosome receptor
RT-PCR	Reverse transcription polymerase chain reaction
RXR	Retinoid X receptor
SAPK	Stress-activated Protein Kinase
SCNT	Somatic cell nuclear transfer
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SFs	Soluble factors
Shh	Sonic hedgehog
SRP	Signal recognition particle

SRPdp	SRP docking protein
SS	Signal sequence
STZ	Streptozotocin
SUR	Sulphonylurea receptor
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TAT	Tyrosine aminotransferase
TBP	TATA-binding protein
TCA	Tricarboxylic acid
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TdO	Tryptophan 2,3-dioxygenase
TF	Transcription factor
TGF	Transforming Growth Factor
TM	Tamoxifen
TRAM	Translocating-chain associated membrane
TRITC	Tetramethylrhodamine isothiocyanate
TTR	Transthyretin
UGT	UDP-glucuronosyltransferase
VEGF	Vascular endothelial growth factor
VGCC	Voltage-gated calcium channel
v/v	Volume for volume
WE	Williams' Medium E
w/v	Weight for volume
x g	gravitational force

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Chapter 1.

Introduction

1.A Endoderm development and organogenesis of liver and pancreas

An important goal for developmental biologists is to understand the steps that allow a single fertilised egg to become a sophisticated organism. This information also has important therapeutic implications since it might allow us to produce specific cell types at will (regenerative medicine). The basic processes for creating lower (worms or flies) and higher (frog, fish, chick, mouse and human) animals are similar (Wells and Melton, 1999). Briefly, a fertilized egg progresses through the formation of the germ layers and body axes (gastrulation and axis patterning), formation of individual organs (organogenesis), and cytodifferentiation to eventually generate a functional organism.

During early development, embryonic cells are divided into the three germ layers (mesoderm, endoderm and ectoderm) through a series of cell movements termed gastrulation. The ectoderm generates the cells of the nervous systems and skin; the mesoderm generates the cells of the connective tissue, muscle, and the circulatory system; and the endoderm gives rise to gastrointestinal organs (oesophagus, stomach, intestines), respiratory tract (lung and trachea), liver and endocrine glands (such as thyroid and pancreas) (Tam and Behringer, 1997). Compared to our knowledge of ectoderm and mesoderm development, the mechanisms underlying endoderm development are more poorly understood. This lack of information is due to a number of reasons. First, most endoderm-derived organs are positioned internally, thus defects associated with endodermal development are rather difficult to observe directly. Second, functions of the endoderm-derived organs including gas exchange, digestion, nutrition absorption, glucose homeostasis and detoxification are vital for maintaining homeostasis in animals. As a result, mutations associated with endoderm

are usually lethal early on in development (Zaret, 1999). However, in the past few years many advances have been made towards elucidating the factors involved in endoderm development. In the following section, the molecular events known to determine competence and patterning of the developing endoderm will be discussed, with particular emphasis on the developmental relationship between liver and pancreas in the mouse.

1.A.1 Early endoderm development

1.A.1.1 Overview of endoderm development prior to gut tube formation

In the mouse, the fertilised egg moves from the oviduct to the uterus around 3 days after fertilisation. During this stage, the fertilised egg becomes a fluid-filled "balloon-like" structure called the blastocyst. A day later, the primitive endoderm emerges from the inner cell mass (ICM). The cells of the ICM are the source of embryonic stem cells (Wells and Melton, 1999). The primitive endoderm contributes to the extraembryonic tissues rather than the formation of the definitive endoderm. Next, during the "egg-cylinder" stage, the blastocyst elongates along the embryonic-abembryonic axis to form a cup shaped structure consisting of two layers, the outer visceral endoderm and an adjacent group of undifferentiated cells, the epiblast. Bone morphogenetic protein (BMP) signalling, specific for BMP2 and BMP4, may regulate the structure of the epiblast and differentiation of visceral endoderm (Coucouvanis and Martin, 1999). This idea is supported by the detection of decreased expression of *Hepatocyte nuclear factor 4* (*HNF4*), a visceral endoderm marker, when BMP signalling is blocked using a transgene encoding a dominant negative mutant

form of BMP receptor. Subsequently, gastrulation begins to form the ectoderm, mesoderm and endoderm from the epiblast.

The primitive streak (PS) is located at the posterior end of the epiblast and appears during gastrulation (around embryonic day 6.5 (E6.5)). The formation of the PS is essential to generate the anteroposterior axis of future embryo (Conlon et al., 1994) and is mediated through the activity of *Nodal* factor (Varlet et al., 1997). Endodermal progenitor cells move out from the anterior PS and migrate towards the distal tip of the epiblast. Several investigations have been carried out to identify the signals that determine the fate of the endodermal cells in the anterior PS. One example of a signal is the requirement for fibroblast growth factors (*Fgfs*). Fgfs to pattern the mesoderm/endoderm cells based on the detection of ectopic neural structures in Fgf receptor-1 deficient mice (Ciruna et al., 1997). By the end of gastrulation (about E7.5 in mice), endodermal cells have acquired regional specification along the anterior-posterior (A-P) axis (Wells and Melton, 1999). There are three lines of evidence to support this statement. First, the genes *Cerberus*, *Otx1*, and *Hesx1* are expressed anteriorly (*IFABP* and *Cdx2* are expressed caudally). Second, during cell migration, cells destined for the anterior move earlier than the cells destined for the posterior end. Third, the posterior endodermal cells divide at a higher rate than the anterior endodermal cells. Differences in patterning along the A-P axis might result from differential signals from the mesoderm (Wells and Melton, 2000).

The next significant event at E8.5 is turning, a process that allows the three germ layers to be correctly positioned within the embryo. The endoderm becomes located inside the embryo while the ectoderm is positioned outside. In addition, the original visceral endoderm now assumes a protective role by surrounding the whole embryo. After turning (E9-E9.5), the gut opening closes and a drastic increase in the growth of

mesenchymal cells around the endodermal gut tube is also observed. After this stage, the embryo proceeds through organogenesis to form individual differentiated internal organs (>E9.5).

1.A.1.2 Signalling factors associated with endoderm formation

Which molecules control endoderm formation? Over the past few decades, much data have been generated allowing these signals to be broadly divided into three categories.

1.A.1.2.1 Signalling factors

Nodal signalling is an extensively studied pathway involved in endoderm formation (Stainier, 2002; Whitman, 2001). *Nodals* are members of the Transforming Growth Factor-β (*TGF-β*) family. One gene has been found in mouse (*Nodal*), two in zebrafish (*cyc* and *sqt*) and five in *Xenopus* (*Xnr1, Xnr2, Xnr4, Xnr5 and Xnr6*). In *Xenopus*, Nodal related molecules induce isolated animal caps to express endodermal genes such as endodermin (Takahashi et al., 2000). Introduction of a dominant negative form of *Nodal* led to the decrease of expression of endoderm-specific genes including *ceberus*, *Xhex1* (a homeobox gene expressed in anterior endoderm) and *Frzb* (a secreted factor expressed in anterior endoderm) (Osada and Wright, 1999; Stainier, 2002). While in fish, the gut endoderm is absent in *sqt;cyc* double mutant embryos (Feldman et al., 1998). The importance of Nodal-related proteins for initiating and patterning embryonic endoderm was further confirmed using knockout mice. Knock-down of *Nodal* activity by Lowe et al suggested that Nodal signalling exhibits a gradient-effect on the formation of mesoderm and endoderm (low – mesoderm; high – endoderm) and this was confirmed in fish (Lowe et al., 2001; Warga and Nusslein-Volhard, 1999). These data

all support the significance of *Nodal* in endoderm development.

Another essential signalling pathway involved in the generation of endoderm is the BMP signalling pathway. Incubation of the BMP antagonist Noggin with *Xenopus* animal caps enhances the expression of the pan-endodermal marker *Endodermin* (Sasai et al., 1996). Furthermore, in *Bmp2* (-/-) and *Bmp7* (-/-) mutant fish, endodermal precursors accumulate (Stainier, 2002).

1.A.1.2.2 Transcription factors

During early development, *Sox, Gata* and *Forkhead* genes are fundamental for initiating endoderm construction. It has been shown that Gata can induce the expression of the intestinal marker *IFABP* in nonendodermal cells both *in vivo* and *in vitro* (Gao et al., 1998). Homozygous inactivation of *FoxA2* in mice results in the absence of the node, the structure that forms foregut and midgut (Ang and Rossant, 1994). *Sox17* plays a highly conserved role in the generation of the endoderm. It is normally expressed in visceral and definitive endoderm and mice deficient for *Sox17* exhibited the lack of axis rotation and deteriorating growth from E8.5 (Kanai-Azuma et al., 2002). Thereafter, disorganised growth and morphogenesis of the posterior trunk was detected, especially after E9.5.

Many transcription factors regulate organogenesis of the endodermal organs (Grapin-Botton and Melton, 2000; Wells and Melton, 1999). In budding organs, investigations using knockout mice have shown that *Pax9* is found to be a master gene for parathyroid formation (Peters et al., 1998); *Nkx2.1* is responsible for thyroid genesis (Kimura et al., 1996); *Gli2/Gli3* (the downstream effectors in Sonic hedgehog (Shh) signalling pathway) are necessary for respiratory organ formation (Motoyama et al., 1998); and *Pdx1* and *Hlxb9* directs development and overgrowth of the pancreatic bud (Jonsson et al., 1994; Li et al., 1999).

Another important event during organogenesis is the enhancement of proliferation in order to allow embryonic organs to reach mature tissue mass. Transcription factors evidenced to be important for this process, including Hlx for intestinal epithelial expansion (Lints et al., 1996), AP-1 and Hepatocyte Growth Factor (HGF) for liver growth (Zaret, 1998) and Cdx1 for proliferation in intestinal crypt cells (Subramanian et al., 1998).

1.A.1.2.3 Inducing factors

The relative position of endoderm to mesoderm during development suggests that interacting signals between these two layers might contribute to organogenesis (Cleaver and Krieg, 2001; Horb, 2000). Some recombination experiments have been carried out to confirm this hypothesis. For example, chick stomach epithelium adopted an intestinal epithelial fate when combined with intestinal mesoderm (Andrew and Rawdon, 1990). Similarly, the lung epithelium failed to branch when cultured with mesenchymal cells of the trachea (Cleaver and Krieg, 2001). More recently, Wells and Melton showed that *in vitro* endoderm explants (i.e. minus the mesoderm and ectoderm) isolated from E7.5 mouse embryos, failed to express *Pdx1*, *Neuro D1* and *Somatostatin*. This observation directly demonstrates that mouse endoderm requires signalling from adjacent ectomesoderm for differentiation. Wells and Melton tested the potential of soluble factors secreted from the mesoderm to regulate endoderm development. The results showed that Fgf4 was able to induce endodermal gene expression in cultured explants (Horb, 2000; Wells and Melton, 2000).

Mesenchymally-derived Fgf signals are also important in specifying other endodermal tissues. For example, Pepicelli et al demonstrated that the initiation of branching in lung epithelium is regulated by Fgf10 secreted from mesenchymal cells (Pepicelli et al., 1998). Conversely, lung development is abolished in *Fgf10* (-/-) mice

(Min et al., 1998). Regarding the regionalisation of hindgut endoderm, Roberts et al showed that endodermal Shh signalling stimulates the expression of *BMP4* and *Hoxd13* in mesoderm and regressively control adjacent endodermal fates (Roberts et al., 1995; Roberts et al., 1998).

The notochord also takes part in patterning the endoderm. The notochord is the first rigid structure formed during early development and functions as a support for the entire embryo. It exists either as a transient structure in higher vertebrates or persists in lower veterbrate (Stemple, 2004). It has been shown that the notochord determines dorsal pancreatic fate through Shh signalling (see section 1.A.3). The importance of the timing of notochord signalling for normal gut development has been demonstrated by the detection of abnormal foregut development after prolonged reception of notochord signals. In addition, the growth deficiency of trachea and oesophagus under sustained contact with the notochord was also observed (Cleaver and Krieg, 2001).

1.A.2 Development of the liver

The parenchymal cells of the liver are derived from the endoderm. The adult liver carries out several essential functions including the secretion of serum proteins, the release of metabolising enzymes and the stabilisation of blood glucose concentration. Therefore, it is not surprising that many regulatory genes are essential for establishing and maintaining these functional properties in embryonic liver cells (Lemaigre and Zaret, 2004; Zaret, 1998; Zaret, 1999; Zaret, 2000; Zaret, 2001; Zhao and Duncan, 2005).

1.A.2.1 Competence stage

FoxA and Gata transcription factors play a highly evolutionarily conserved role in endoderm patterning, as demonstrated by deficient endoderm development in knockout animals, from mammals to worm (Gaudet and Mango, 2002). Recently, Zaret's group proposed a model stating that FoxA2/Gata4 (potentiation factors), can "pre-pattern" the hepatic endoderm at around embryonic stage E6-E7. This model was demonstrated by sophisticated techniques, chromatin immunoprecipitation and *in vivo* footprinting, to determine the occupancy of sites in the albumin promoter by FoxA2 and Gata4 proteins at an early embryonic stage (Bossard and Zaret, 1998; Cirillo et al., 2002). The FoxA binding site is occupied before the albumin gene is expressed (before E8). The occupancy persisted from E8.5-E11.5 and additional recruitment of other transcription factors including CCAAT/enhancer-binding protein β (C/EBPβ) and Nuclear Factor 1 (NF1) is needed for the active expression of the albumin gene. This early DNA-protein interaction suggested that FoxA and Gata4 may determine the hepatic endodermal cell fate after gastrulation and enhances it in combination with more liver-specific transcription factors.

1.A.2.2 Specification and proliferation stage

Induction of the hepatic fate is thought to occur via repression of the default pancreatic programme by signals secreted from neighbouring mesenchymal cells (Deutsch et al., 2001). Between E7.5-E9, the cardiac mesoderm directly interacts with hepatic endoderm and hepatic differentiation begins. Some soluble factors, such as Fgfs, mediate regulation of liver development as evidenced by the induction of *α1-fetoprotein* (*AFP*) and *albumin* mRNAs by treating the isolated foregut endoderm tissues with Fgfs (Jung et al., 1999). Additionally, another mesenchymal tissue surrounding the hepatic endoderm, the septum transversum, is essential for

hepatocyte specification through secretion of BMPs, mainly BMP2 and BMP4 (Rossi et al., 2001). At about the same stage, *Hex* (Haematopoietically expressed homeobox) is also involved in normal liver development since *Hex(-/-)* mice exhibit impaired liver growth (Martinez Barbera et al., 2000). In addition to signalling from cardiac mesoderm, non-mesenchymal endothelial cells are also important for liver proliferation and migration. Matsumoto et al. showed this both *in vivo* (*Vascular Endothelial Growth Factor Receptor 2* (*VEGFR2*) deficient mice) and *in vitro* (embryonic liver bud cultures treated with angiogenesis inhibitor NK4 to block vessel formation) models (Matsumoto et al., 2001). In *VEGFR2* knockout mice, the early hepatocytes formed a multilayered epithelium but failed to form specified liver cells while in the NK4-treated embryonic liver bud cultures impaired liver buds growth was observed. These data confirmed the role of endothelial cells on early liver organogenesis (Nikolova and Lammert, 2003).

After the cell fate was determined, the hepatic endoderm is pulled to the midgut and early liver cells begin to proliferate within the endoderm at 9.5 days after fertilisation. At this stage, the liver mass is dramatically increased and haematopoietic cells migrate into the liver. The rod-like hepatoblasts secrete extracellular degrading enzymes to dissolve the basement membrane and migrate into the septum transversum (Michalopoulos and DeFrances, 1997). Several factors are essential for early liver development including Prox-1. *Prox-1* (Prospero-related homeobox 1) is a homeobox gene originally cloned by homology to the *Drosophila melanogaster* gene *prospero*. It is expressed mainly in the adult central nervous system and in the developing eye lens, ventral pancreas, heart and liver (Oliver et al., 1993). Homologous knockout *Prox-1* mice exhibit a defect in migration of liver cells at E10 due to the presence of an abnormal basement membrane rich in E-cadherin, type 4 collagen and laminin (Sosa-Pineda et al., 2000). This observation indicated that

Prox-1 is crucial for hepatocyte delamination into the septum transversum. Gene disruption experiments also found that transcription factors associated with proliferation and apoptosis such as c-jun, RXR (Retinoid X receptor), jumonji, N-myc, K-ras and Rel A (NF-κB subunit) are also indispensable for differentiation and growth of the hepatocyte population (Zaret, 1998).

In addition to the soluble factors regulating the specification of early liver cells, the septum transversum also provides some signals to the developing liver to promote hepatocyte outgrowth and differentiation. Some evidence from gene-silencing studies showed that hepatocyte growth factor (HGF) signalling participated in this process. Hlx, a mesenchymal-specific transcription factor, activates the expression and secretion of HGF in the septum transversum. In turn, HGF activates the tyrosine kinase receptor c-met located on the hepatoblast cell surface (Jiang et al., 2005). Consequently, the phosphatidylinositol-3 kinase (PI3K), mitogen-stimulated STAT signalling or stress activated JNK kinase pathway is activated leading to the expression of the proliferation-related complex AP-1. In addition to the interacting receptors between cells and the extracellular matrix, *integrin* families also play a role in regulating the balance between proliferation and apoptosis during hepatocyte formation (Lora et al., 1998).

1.A.2.3 Hepatocyte or bile duct?

During liver outgrowth, hepatoblasts face a decision to become either hepatocytes or biliary cells (Suzuki et al., 2002). Recent evidence suggested that molecules including signalling factors and transcription factors are fundamental to distinguish hepatocyte and bile duct cell fates (Lemaigre and Zaret, 2004; Zhao and Duncan, 2005). TGF-β, Notch and Wnt are all possible candidates involved in the control of bile duct cell formation. For example, heterozygosity of the Notch ligand

Jagged 1 leads to the clinical autosomal dominant disorder, Alagille syndrome, characterised by the loss of the intrahepatic bile ducts in humans (Lemaigre and Zaret, 2004). Coincidently, the Notch component Delta-like protein is expressed at the same time hepatoblast fate is determined (~E10.5) (Tanimizu et al., 2003). In *Hes1* (-/-) mice, extrahepatic bile ducts are converted to both exocrine and endocrine pancreatic cell types. A role for Notch signalling in ductal cell determination is further supported by the observation that Hes1 is a negative regulator of the pro-endocrine gene *Neurogenin 3 (Ngn3)* as well as the downstream gene in the Notch signalling pathway (Sumazaki et al., 2004). In addition, the expression of various HNF networks plays a significant role in specifying the decision between hepatocyte and bile duct cell fate of hepatoblasts. HNF6 and HNF1β are the two main transcription factors regulating bile duct formation. Biliary differentiation occurs prematurely in *HNF6* knockout mice and the same defect is also detected in mice with liver-specific deficiency of *HNF1β* (Clotman et al., 2002; Coffinier et al., 2002). In contrast, conditional loss of function of *HNF4α* indicated the requirement of HNF4α in hepatocyte maturation and epithelialisation (Parviz et al., 2003). Thus, the HNF networks actively regulate the latter stage of liver cell development (Odom et al., 2004).

1.A.3 Development of the pancreas

In the recent years, considerable understanding of pancreas development has been gained. The pancreas is composed of three major functional units: the exocrine cells that secrete digestive enzymes for the digestion of macromolecules into smaller molecules; the duct cells that mediate transportation of digestive enzymes from the exocrine cells to the small intestine; and the endocrine cells that synthesise and secrete hormones. The exocrine and ductal cells represent more than 95% of total cell

populations in the pancreas. About 2% is comprised of at least four different types of endocrine cells including α, β, δ, PP cells which produce glucagon, insulin, somatostatin and pancreatic polypeptide, respectively (Slack, 1995). More recently, another endocrine cell (called ζ-cells) was discovered in the pancreas. This cell type secretes the hormone ghrelin and is involved in the regulatory network of energy balance by increasing food intake and reducing fat utilization (Chanoine, 2005; Kojima et al., 1999; Wierup et al., 2002; Wierup et al., 2004). Organogenesis of the pancreas is discussed below with particular emphasis on the molecules that determine the endocrine cell fate.

1.A.3.1 Pancreatic organogenesis

1.A.3.1.1 Commitment to pancreatic fate

The commitment to a pancreatic fate occurs as early as E8.5 in mouse. Several signals released from adjacent structures including notochord, aorta and cardiac mesoderm are thought to be essential for the patterning of pancreatic endoderm (Wells and Melton, 2000). Inhibition of Shh signalling is required for patterning the dorsal pancreatic endoderm. This idea is supported by the detection of smooth muscle-like mesenchyme and interstitial cells of Cajal, characteristic of the intestine, rather than pancreatic mesenchyme in the pPdx1-*Shh* transgenic mice (Shh signals driven by Pdx1 promoter) (Apelqvist et al., 1997). A similar program of intestinal differentiation was also observed when the pancreatic explants were exposed to Shh (Apelqvist et al., 1997). Conversely, loss of *Shh* provokes a relative increase in endocrine cell number and pancreas size (Hebrok et al., 2000). It has been shown that activin-βB, a member of TGF-β family, and Fgf2 secreted from the notochord are able to repress the expression of *Shh* therefore activating the pancreatic programme

in dorsal pancreatic endoderm (Hebrok et al., 1998; Kim et al., 1997). Interestingly, presence of Shh rather than absence of Shh, is required for pancreatic development in Zebrafish (Roy et al., 2001) indicating that in this regard the fish is very different from higher vertebrates.

The ventral pancreas is determined by the signals derived from the cardiac mesoderm, namely Fgfs and BMPs (Deutsch et al., 2001). The relative position of the endodermal epithelium and blood vessels also revealed a possible role of signals from vascular endothelium on pancreas development. At E9.5, the dorsal pancreatic bud is located adjacent to the aorta while the ventral pancreatic region is positioned close to the vitelline veins (Lammert et al., 2001). It was demonstrated that the signal from vessels, probably mediated through the activity of vascular endothelial growth factor (VEGF) plays a fundamental role on pancreatic differentiation (Lammert et al., 2001). The development of the ventral and dorsal pancreatic buds is quite different. For example, the onset of mesenchymal condensation is different in the dorsal (E9.5) and ventral (E10.5) buds (Herrera et al., 2002). In addition, different phenotypes are induced in the dorsal and ventral pancreas in knock-out animals (*Isl1(-/-)*, *N-cadherin(-/-)* and *Hlxb9(-/-)*) (see table 1.1) suggesting that the molecules determining dorsal and ventral pancreas are distinct.

Targeted gene	Dorsal pancreas	Ventral pancreas
Pdx1	Aplasia	
Ngn3	No islets	
PTF1-p48	No exocrine cells	
Pax4	No β or δ cells	
Pax6	No α cells; endocrine hypoplasia	
Isl1	Agenesis; no mesenchyme	No endocrine cells
Hlxb9	Agenesis	Fewer β cells
Hes1	Hypoplasia	
N-cadherin	Agenesis; no mesenchyme	Normal
Beta2/NeuroD	Fewer β cells; distorted islets	
Hnf6	Endocrine hypoplasia	
Pbx1	Hypoplasia	
Nkx2.2	Immature β-cells	

Table 1.1 Phenotype of dorsal and ventral pancreas in knockout mice of various pancreatic transcription factors. Reproduced from Herrera et al 2002

1.A.3.1.2 Timing of appearance of different cell types and later events

Morphogenesis of the pancreas commences at about E9.5 and is first observed by the condensation of the dorsal mesenchyme. Both dorsal and ventral buds subsequently proliferate and undergo extensive branching. The endocrine cells appear at different times during development. Using immunofluorescent staining, glucagon-expressing cells are first detected at E9.5 in the dorsal pancreas (E10.5 in ventral pancreatic bud), followed by insulin and somatostatin expressing cells at E10.5-E11.5 and E13.5, respectively. The exocrine cells start differentiating from E14.5, while pancreatic polypeptide expressing cells are not detected until E18.5 (Herrera et al., 1991; Slack, 1995). However, RT-PCR analysis performed on mouse tissue revealed the presence of *glucagon* and *somatostatin* mRNAs as early as E8.5-E9. At E10.5, the *insulin I* gene and *PP* mRNAs were found and at E11.5, *insulin II* was detected (Gittes and Rutter, 1992; Herrera et al., 1991). The early endocrine cells are located within the ductal epithelium and may contain more than one hormone in each cell (e.g. glucagon and PP- have been found co-localised in the same cells at

E10.5) (Herrera et al., 1991). Later the cells interact with adhesion molecules, including collagen IV, to migrate into the surrounding connective tissues and form clusters (Herrera et al., 2002). At about E17, gut rotation allows the ventral pancreatic buds to fuse with the dorsal bud thus generating a single functional organ (Edlund, 2001; Edlund, 2002; Grapin-Botton et al., 2001) and one day later the typical islet is found with the "one cell-one hormone" cell types (Herrera et al., 2002).

1.A.3.2 Cellular origins of pancreatic cell types

The question arises as to whether pancreatic cells are derived from one single origin (monoclonal) or numbers of progenitors (polyclonal)? To answer the question about cell origin, lineage tracing experiments were performed to label the progenitors and trace their descendants. Early methods used vital dyes, such as lipid-soluble carbocyanine dyes DiI or DiO to tag the cells. Due to their hydrophobic properties, the dyes become integrated into the cell membrane and hence label the cells. However, there are two problems associated with this technique. The first is that the dye labels groups of cells rather than individual cells so this makes it impossible to elucidate the fate of an individual cell. The second concerns the dilution of labelled cells during cell division (Herrera et al., 2002).

To overcome these disadvantages, genetic marking methods have been employed. One of the most convincing and commonly used methods is the Cre/loxP system (Rajewsky et al., 1996). This system is based on the idea that the progenitors can be labelled with a reporter gene after the excision of a *LoxP-stop-LoxP* genetic cassette by a cell- or tissue-specific Cre recombinase (Gu et al., 2003). Using this technique, the origin of pancreatic duct, exocrine and islet cells was addressed (Gu et al., 2003; Herrera et al., 2002). It was shown that: (a) all pancreatic cells are derived from endodermal epithelium and differentiated from Pdx1-expressing

precursors(Herrera, 2000; Percival and Slack, 1999); (b) PTF1a-p48 is involved in determining both exocrine and endocrine pancreatic lineages (Kawaguchi et al., 2002); (c) islet endocrine cells originate from Ngn3 positive progenitor cells (Gu et al., 2002); (d) the insulin and glucagon cells develop independently (Herrera, 2000; Jensen et al., 2000a) and (e) exocrine, endocrine and ductal lineages are separated by E12.5(Grapin-Botton, 2005).

1.A.3.3 Hierarchy of transcription factors regulating different pancreatic cell fates

In contrast to the studies of the factors controlling the exocrine and ductal cells, the regulation of pancreatic endocrine cell formation has been much more extensively investigated. Greater interest in endocrine pancreas arises from the possible application to the treatment of diabetes (Weir, 2004; Weir and Bonner-Weir, 2004). The investigations based on gene-knockout showed that many factors, mainly the transcription factors located on the *cis*-regulatory region of endocrine hormone (i.e. insulin), are involved in the regulation of the pancreatic endocrine cell development. Basically, these transcription factors belong to three different families: the homeodomain family, the basic helix-loop-helix (bHLH) family and the winged helix family of proteins (Schwitzgebel, 2001). The following paragraphs introduce these transcription factors including the onset of expression, function and the studies performed showing the roles of these factors in the developing pancreas (see Figure 1.1) (Wilson et al., 2003).

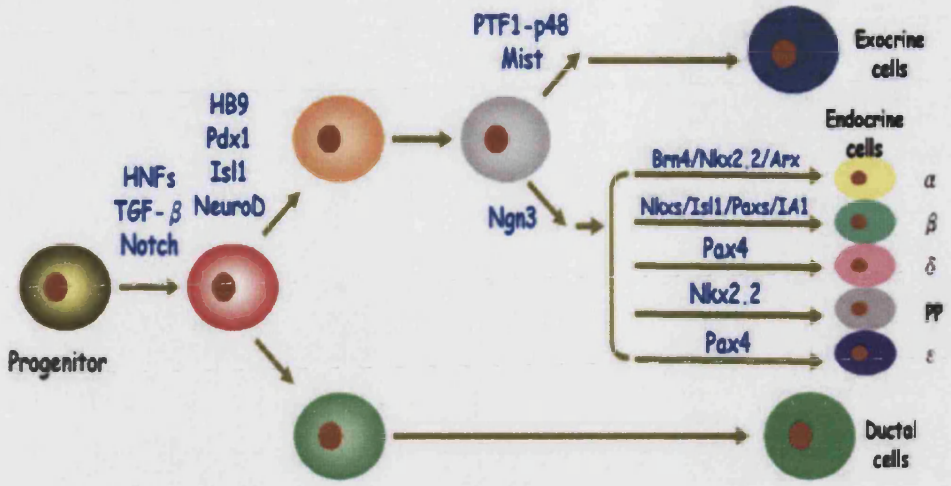


Figure 1.1 A simplified model for the role of islet transcription factors in endocrine differentiation in the developing pancreas.

1.A.3.3.1 Forkhead box (Fox) A family (Hepatocyte nuclear factors 3, HNF3)

Foxa1 (HNF-3α) and Foxa2 (HNF-3β) belong to the winged helix family of transcription factors. These transcription factors contain a Fox DNA-binding domain along with four transactivation domains (Kaestner, 2000). As described in section 1.A.2, FoxA2 is expressed at about E6-E7 and is essential for formation of the foregut endoderm. Since homozygous deletion of the Foxa2 gene leads to the embryonic lethality at E11.5, due to a defect in foregut morphogenesis, a conditional knockout is needed to investigate the function of Foxa2 in pancreas development. Recently, a specific knockout of *Foxa2* in pancreatic β cells was described. The animals showed hyperinsulinemic hypoglycemia revealing that Foxa2 is important for the maintenance of normal pancreatic histoarchitecture and the preservation of a normal physiological response to glucose. More recently, it was suggested that Foxa2 is also essential for the generation of glucagon-producing cells (Lee et al., 2005; Sund et al., 2001). *Foxa1* is expressed in the endoderm at E7.5 (Monaghan et al., 1993) and knockout of *Foxa1* does not affect development of the pancreas. Postnatally, targeted disruption of

Foxa1 in the pancreas led to hypoglycaemia due to the reduced transcription of the glucagon gene (Kaestner et al., 1999).

1.A.3.3.2 Hlxb9

The *Hlxb9* gene is a homeobox gene encoding HB9 protein. The earliest expression of *Hlxb9* is detected at E8 in the notochord and gut endoderm and is later restricted to the adult islet β cells (Li et al., 1999). Deficiency of *Hlxb9* in mouse pancreas causes failure of dorsal pancreas formation. The ventral pancreas forms at the expected time but with reduced β cell population (Li et al., 1999). It has been suggested that *Hlxb9* is required for the generation of the dorsal pancreas as well as the terminal differentiation and maturation of pancreatic β cells. Conversely, experiments using the Pdx1 promoter to drive expression of *Hlxb9* in the pancreas resulted in drastically perturbed pancreatic organisation and impaired differentiation. This notion is evidenced by the detection of more elongated, tube-shaped intestinal-like structure in the pancreatic area and decreased insulin-, amylase- and carboxypeptidase A-expressing cells (Li and Edlund, 2001). It further confirmed the necessity of tightly-controlled temporal expression of *Hlxb9* during pancreas development.

1.A.3.3.3 Pancreatic duodenal homeobox 1 (Pdx1)

Pdx1 is a homeodomain containing protein and may be the most widely-studied transcription factor involved in pancreas development. Pdx1 is expressed in the pancreatic primordium, distal stomach epithelium and proximal duodenal region at around E8.5 (Guz et al., 1995) and has been shown important for the transactivation of several pancreatic genes including *insulin*, *glucagon*, *somatostatin*, *islet amyloid polypeptide* (*IAPP*) and glucose transporter *GLUT-2* (Miller et al., 1994; Ohlsson et al.,

1993; Waeber et al., 1996; Watada et al., 1996a). In mice carrying a null mutation for the *Pdx1* alleles, the pancreas was absent, despite the initial formation of the pancreatic bud (Ahlgren et al., 1996; Johansson et al., 1999; Offield et al., 1996). Based on this result, the function of Pdx1 in later stages of pancreas development could not be examined. Recently, mice were generated with *Pdx1* expression disrupted specifically in the pancreatic β cells. The mice lost β cells and developed diabetes. This demonstrated a second role for Pdx1 in the maintenance of pancreatic β cell identity and glucose-sensing machinery (Ahlgren et al., 1998). More recently, a mouse model was generated in which *Pdx1* can be switched on or off at a specific stage of pancreas development. The results suggested that Pdx1 is essential for maintenance of β-cell functions, correct pancreatic structure, as well as exocrine development in the adult pancreas (Hale et al., 2005; Holland et al., 2005).

1.A.3.3.4 Islet 1 (Isl1)

Islet 1 belongs to the LIM-homeodomain family of proteins. In the pancreas, expression of *Isl1* begins at E9 in the dorsal pancreatic epithelium and the mesenchymal region surrounding the dorsal bud but not in the ventral pancreatic primordia. Later, *Isl1* is detected in the adult islets (Ahlgren et al., 1997). In *Isl1* mutant mice, development is arrested soon after E9.5. The dorsal pancreatic mesenchyme is not formed, endocrine development is abolished and the exocrine component of the dorsal pancreas is largely decreased. However, the exocrine cell deficiency was not seen in the ventral region. The importance of Isl1 in islet cell differentiation was further confirmed by explant experiments. Exocrine, but not endocrine, cell differentiation in the dorsal pancreas can be rescued *in vitro* by co-culture with the mesenchyme derived from wild type embryos suggesting that Isl1 is essential for the generation of the dorsal pancreatic mesenchyme which in turn is important for exocrine

differentiation. In addition, Isl1 has been shown to bind the promoters of several pancreatic genes including *insulin*, *glucagon* and *somatostatin* (Brink, 2003) and is therefore necessary for the functional identity of islet cells.

The aforementioned transcription factors mainly play a role in determining the region of the gut endoderm to become the pancreas (Pdx1 is also required for β-cell function). Sequentially, the following factors are responsible for defining the formation of exocrine or endocrine progenitors (such as Ngn3, PTF1a-p48 and HNF6) and are required for the final differentiation of the various cell lineages.

1.A.3.3.5 Hepatocyte nuclear factors 6 (HNF6)

During pancreas development, *HNF6* is detected at E9.5 in the epithelial cells and is later restricted to the exocrine and ductal cells (Rausa et al., 1997). In *HNF6 (-/-)* embryos, the exocrine pancreas develops normally however the endocrine phenotype is severely inhibited. At birth, fewer endocrine cells were found and the islets of Langerhans are absent in this animal. Interestingly, the islets reappeared with disrupted architecture at a later stage (Jacquemin et al., 2000). Therefore, the pancreatic epithelial cells can be committed in the absence of HNF6. However, they may fail to give rise to the expected precursor endocrine pool. This notion is supported by reduced *Ngn 3*, a potential marker of endocrine precursor cells, expression in *HNF6* null mice (Jacquemin et al., 2000).

1.A.3.3.6 Ngn 3 and Hairy/Enhancer of Split-related protein 1 (Hes-1)

Ngn3, a bHLH transcription factor, is expressed in pancreas from E9.5, peaks at E15.5 and diminishes thereafter to undetectable levels in the neonatal mouse pancreas (Apelqvist et al., 1999). The *Ngn3* knockout animal lacks endocrine cells at

birth, while the persistent overexpression of *Ngn3* drives the formation of additional endocrine cells (Apelqvist et al., 1999; Gradwohl et al., 2000). It is thought that Ngn3 is responsible for the commitment of endocrine cells rather than all pancreatic cell lineages and mirrors its role in the later stages of pancreas development. It further confirmed that Ngn3 may require another 'central regulator' to change cell fate. More recently, Ngn3 was also shown to be crucial for differentiation of endocrine cell types in intestine and stomach (Jenny et al., 2002). For example, Ngn3 might be a mediator for converting intestinal epithelial cells to insulin-positive cells following treatment with glucagon-like peptide 1 *in vitro* and *in vivo* (Suzuki et al., 2003).

Hes1 also encodes a bHLH protein and is expressed at E9.5 in pancreas. The detection of pancreatic endocrine cell excess in the *Hes1* (-/-) animals has suggested that Hes1 may have a role in repressing endocrine cell development through Notch signalling (Jensen et al., 2000b). The elevated expression of Ngn3 in the stomach, small and large intestine in *Hes1* (-/-) mice confirmed that Hes1 and Ngn3 have opposite effects on the determination of endocrine cell fate (Sumazaki et al., 2004). Based on the pro-endocrine action of Ngn3, impaired expression of Hes1 would be predicted to increase differentiation of pancreatic endocrine cells. To further support this idea, conversion of the developing biliary system to pancreatic tissue in *Hes1* deficient mice has recently been described (Burke et al., 2004; Sumazaki et al., 2004). The experiments also claimed that the conversion from biliary system to pancreatic-like cells might be through the re-activation of Ngn3 that is initially restrained by Hes1.

The generation of scattered endocrine islets in the pancreas from an initial homogenous field of cells is executed by a process termed lateral inhibition mediated by the Notch signalling pathway. Along with the observations of *Ngn3* and *Hes1* deficient animals, a model about how the exocrine-endocrine cell fate is determined

was proposed (Docherty, 2001; Edlund, 2001). In this model, the high expression of Ngn3 activates Delta, the Notch ligand, in the endocrine cells and stimulates the adjacent cells to upregulate Hes1, in turn inhibiting Ngn3 expression to make the neighbouring cells pursue a non-endocrine fate (see Figure 1.2) (Docherty, 2001; Jensen, 2004).

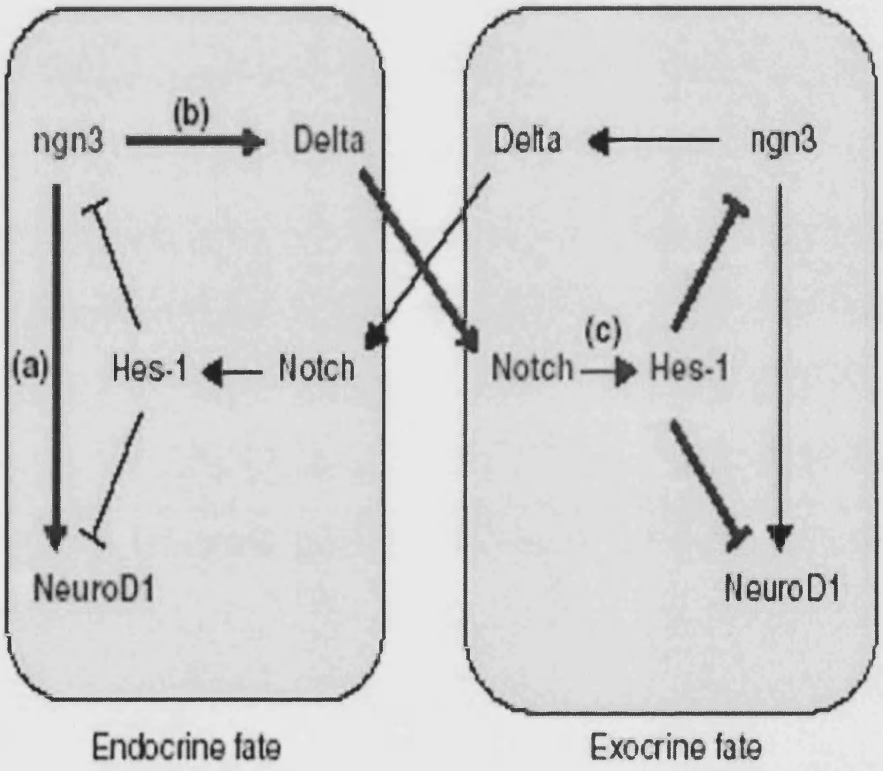


Figure 1.2 Role of Delta/Notch signalling in controlling endocrine versus exocrine cell differentiation. Reproduced from Docherty 2001

1.A.3.3.7 Pancreatic transcription factor 1 (PTF1-p48)

The bHLH transcription factor PTF1-p48 was first described as a DNA-binding subunit of the hetero oligomeric trancription factor PTF-1 The latter governs the expression of genes in the exocrine pancreas (Krapp et al., 1996). Recent results have revised the role of PTF1-p48, to also include an involvement in both exocrine cell lineage determination and accurate endocrine spatial organization in murine and

zebrafish models (Kawaguchi et al., 2002; Krapp et al., 1998; Lin et al., 2004).

1.A.3.3.8 Prox1

In addition to the importance for liver development, the homeobox gene Prox1 has been shown to be expressed at E8.5 in the endodermal region that will give rise to liver and pancreas (Burke and Oliver, 2002). The fundamental role of Prox1 for pancreas growth and appropriate morphogenesis (i.e. branching) has been confirmed recently using *Prox1* (-/-) mice (Wang et al., 2005). Prox1 possesses a second role for further differentiation of endocrine cells based on the observation of substantial decrease in Ngn3, Pax4, NeuroD1 and insulin producing cells at E13.5 in gene-disrupted mice (Wang et al., 2005). Conversely, the pancreatic exocrine cell numbers were shown to be increasing. Altogether, these observations suggest Prox1 might not be involved in initiating pancreas development but it may be essential in stabilising endocrine cell identity.

1.A.3.3.9 NeuroD1 / β cell E-box trans-activator 2(Beta2)

NeuroD1 is a bHLH transcription factor expressed in the developing pancreatic epithelium at about E9.5 and in β cells throughout development (Naya et al., 1995). The essential role for NeuroD1 in pancreatic endocrine development was shown by the detection of severely reduced endocrine phenotypes in homozygous mice at birth. *NeuroD1* (-/-) mice died within 5 days after birth due to the hyperglycaemia resulting from impaired islet formation. Moreover, β cells undergo massive cell death starting around E17.5 in *NeuroD* mutant mice (Chae et al., 2004; Naya et al., 1997).

1.A.3.3.10 Paired-box homeobox gene Pax4/6

The paired-box homeobox genes encode key transcription factors involved in the organogenesis of many tissues including eye, brain and endoderm-derived organs such as the pancreas and thyroid gland (Mansouri et al., 1999). Among them, Pax4 and Pax6 are associated with pancreatic endocrine differentiation. *Pax4* and *Pax6* are first expressed in the pancreas at E9.5 and E9, respectively (Sosa-Pineda et al., 1997; Turque et al., 1994). Targeted-disruption of *Pax4* results in the absence of β and δ cells in the newborn mice whereas α cells are increased (Sosa-Pineda et al., 1997). The insulin and glucagon producing cells were detected in mutant mice at E10.5, however, insulin is no longer present by E13.5. Therefore, Pax4 plays a similar role to Prox1, that is to say, to maintain β cell differentiation and maturation rather than to initiate it. On the other hand, Pax6 seems to be more crucial for the α cells (glucagon-producing cells)(Sosa-Pineda, 2004; St-Onge et al., 1997). Double knockout of *Pax4* and *Pax6* have also been generated and show the complete loss of endocrine cell types (St-Onge et al., 1997). Hence, Pax4 and Pax6 act cooperatively to regulate islet cell identities.

1.A.3.3.11 NK class homeodomain protein Nkx2.2/Nkx6.1

NK class homeobox genes, Nkx2.2 and Nkx6.1, act at a relatively late stage of pancreas development. *Nkx2.2* is expressed at E9.5 in the whole pancreatic endocrine epithelium then becomes restricted to α, β and PP cells (Sussel et al., 1998). Reduced expression of *Nkx2.2* decreases the number of (i) α and PP cells and (ii) the number of mature β cells (as indicated by the detection of IAPP and prohormone convertase 1/3 (PC1/3) in the absence of insulin). More recently, Prado et al. found that β cells were replaced by pancreatic ε cells (ghrelin-positive cells) in both *Nkx2.2* (-/-) and *Pax4* (-/-) mice (Prado et al., 2004).. The expression of Nkx6.1

protein starts at E10.5 broadly in whole pancreatic epithelium and becomes confined to insulin producing cells in the adult pancreas (Oster et al., 1998). *Nkx6.1* null animals display profoundly decreased β cell numbers after E12.5 stage (Sander et al., 2000). Therefore, Nkx6.1 is required for expansion and final differentiation of β cell progenitors.

The phenotype of the *Nkx2.2* (-/-) and double (*Nkx2.2* and *6.1*) knockout animals is very similar (Sander et al., 2000). Since Nkx2.2 is still present in *Nkx6.1* (-/-) mice this indicates a possible common pathway with Nkx2.2 upstream of Nkx6.1.

1.A.3.3.12 Other transcription factors

In addition to the factors mentioned above, some additional transcription factors have been found to be important for pancreas development. For example, a POU-homeodomain protein Brn4 was found to be expressed in the developing pancreas and is responsible for the determination of progenitors to pancreatic α cell lineage (Heller et al., 2004; Hussain et al., 2002). In addition, the homeobox containing transcription factor Arx, which has restricted expression in the islet cells of the adult pancreas, is also required for α cell generation (Collombat et al., 2003). Recent data suggested that Arx might be cooperatively interacting with Pax4 thereby mediating proper endocrine fate allocation (Collombat et al., 2005).

1.B Transdifferentiation

1.B.1 Overview

It used to be generally accepted that the terminally differentiated state of a cell was fixed. However, we now know this is not the case and it has become clear that the differentiation status of some cell types can be reversed (Slack, 1985; Slack, 1986; Tosh and Slack, 2002). For example, the ectopic appearance of unexpected cell types during embryogenesis or regeneration does occur and is referred to as "homeotic transformation", heterotopias or transdetermination (Slack, 1985). Most homeotic transformations are observed among the *Arthropoda* or *Crustacea* but it has also been documented in humans (Slack, 1985). Metaplasia is the term used to describe a change in tissue type which occurs in postnatal life (Slack, 1985) and can also be used to represent the alteration in cell fate of stem cell populations (Slack and Tosh, 2001). Transdifferentiation is one subset of metaplasias defined as an irreversible switch of one type of already differentiated cell to another, although such events are believed to be extremely rare in nature (Tosh and Slack, 2002). Normally dedifferentiation and cell division are essential intermediate processes for transdifferentiation although they may not be obligatory in all cases (Beresford, 1990). Transdifferentiation and metaplasia are associated with a discrete change in cellular morphology as well as a change in the programme of gene expression. At the molecular level, the cause of transdifferentiation is presumably a change in the expression of a master regulatory (master switch) gene whose normal function is to distinguish the two tissues in normal embryonic development (Slack and Tosh, 2001; Tosh and Slack, 2002).

It is important to study transdifferentiation for several reasons. First,

understanding the molecular basis of cell-type switches will enhance our knowledge of normal developmental mechanisms. Second, from a pathological point view, some metaplasias predispose to malignant cancer and therefore may reveal useful cellular or molecular information for developing treatments for these diseases. Third, perhaps of most interest at present, uncovering the mechanisms controlling cell type conversions improve the ability to reprogramme cell fates for the development of therapeutic strategies for the treatment of degenerative and autoimmune disorders, such as Parkinson's disease and diabetes mellitus (Burke and Tosh, 2005; Kume, 2005) .

1.B.2 Criteria and Controversies of transdifferentiation

In order to clearly demonstrate that transdifferentiation has occurred, two criteria must be fulfilled (Eguchi and Kodama, 1993; Slack and Tosh, 2001; Tosh and Slack, 2002). The first prerequisite is that the differentiated state before and after transdifferentiation must be identifiable. Morphological differences between parental and transdifferentiated cells can be used to achieve this goal but further biochemical or molecular evidence should also be demonstrated. For example, functional assays specific for the converted cell type could be performed. The second requirement is to show the direct ancestor-descendant relationship between the cell types before and after the switch. One commonly used approach to address the ancestor-descendant relationship is to use the Cre-lox system. By this means, the progenies from the original cell type can be permanently labelled and therefore enable direct demonstration of the conversion event.

Recently, the term "transdifferentiation" has been applied to experiments of

grafting of haematopoietic stem cells, or other stem cell types, into host animals. Some workers have claimed to find extensive colonisation by donor cells of differentiated tissues in the host and called this transdifferentiation. This claim has been highly controversial (Wells, 2002). One argument against this phenomenon is that, rather than direct conversion, cell-fusion may account for the mechanism behind the cell fate change. Alvarez-Dolado et al. found evidence of fusion of bone marrow derived cells (BMDC) with neurons, hepatocytes and cardiomyocytes *in vivo* (Alvarez-Dolado et al., 2003). In addition, there is also the example of BMDC incorporation into fully differentiated, non-proliferative Purkinje neurons in both humans and mice (Weimann et al., 2003a; Weimann et al., 2003b). Cell fusion in non-dividing cells or damaged tissues, such as Purkinje neurons and cardiomyocytes, may still prove useful for developing cell therapies, however, these results did negate the transdifferentiation theory. Further evidence from transplantation studies demonstrates that hematopoietic stem cells (HSCs) can rescue liver function in a mouse model of hereditary tyrosinaemia type I (Lagasse et al., 2000). These mice lack the enzyme fumaryl-acetoacetate hydrolase (FAH) and can only survive if treated with the drug 2-(2-nitro-4-trifluoro-methylbenzyol)- 1,3-cyclohexanedione (NBTC). HSCs derived from *Fah* (+/+) mice reconstituted the *Fah* (-/-) liver by fusing with the existing hepatocytes. The authors excluded the possibility that HSCs from *Fah* (+/+) mice converted to hepatocytes prior to fusion (Wang et al., 2003). It was also shown that granulocyte macrophage progenitors (GMP, the colony forming units of progenitor for granulocytes, macrophages and dendritic cells) or bone marrow-derived macrophages fuse with the host hepatocytes and rescued the mutant phenotype when transplanted into *Fah* (-/-) mice (Willenbring et al., 2004). In addition to these *in vivo* models, it has been also demonstrated that mouse bone marrow derived cells can spontaneously fuse with embryonic stem cells when cultured in the presence of

the cytokine interleukin-3 (IL-3) (Terada et al., 2002). Ying et al. also found that neural stem cells co-cultured with embryonic stem cells could alter the phenotype through spontaneous generation of hybrid cells (Ying et al., 2002).

To date, BMDCs, consisting of HSCs and mesenchymal stem cells (MSCs), have been shown to fuse with liver, skeletal muscle, cardiac muscle and neurons to generate target tissues (Pomerantz and Blau, 2004). It is important to distinguish between the mechanisms of direct transdifferentiation versus cell fusion. One way to define these possibilities, in most cell types, is by the determination of the number of nuclei or chromosomes in transdifferentiated cells (Ambrosi and Rasmussen, 2005). There is evidence that fused cells become mononucleated again, either by nuclear fusion or by supernumerary nuclei elimination (Alvarez-Dolado et al., 2003; Wang et al., 2003). Whether the fusion products, the stable heterokaryons, can reactivate post-mitotic, terminally differentiated cell types to undergo cell division remains to be investigated. Furthermore, tissue damage is thought to be a primary factor for BMDC fusion with target cells, however, the precise signals regulating fusion and why only some cell types can fuse and others not is still unclear (Alvarez-Dolado et al., 2003; Wells, 2002).

Undoubtedly, more unambiguous carefully-designed experiments to confirm whether or not the transdifferentiation is a real biological event are required. In addition, how to amplify this event using molecular engineering or environmental selection might be another important issue for the application of transdifferentiated cells onto the therapeutic points (Wells, 2002).

1.B.3 Identification of master switch gene(s)

During embryogenesis, different tissue types arise from a common cell sheet. By

the orchestration of an on/off state of various regulatory factors in a specific temporal and spatial manner, individual tissues are formed (Soria, 2001). Since transdifferentiation appears to be the result of a single-step change, it is logical to assume that tissues between which such changes occur are neighbours in the sense that the combination of selector genes that defines them differ only in the state of one gene or a few genes (Slack and Tosh, 2001). The factors involving the stabilisation of the differentiation state might also be key regulators controlling cell fates or cell type switch (Eguchi and Kodama, 1993). For instance, the striated muscle tissues in jellyfish can be converted into neurons but transdifferentiation is inhibited in the absence of the extracellular matrix (ECM) (Schmid et al., 1993). This indicated that transdifferentiation is a cell-ECM interaction dependent event. In addition to the factors embedded in cell substrate, a number of genes, especially transcription factors, exemplify the definition of a master switch gene. For example, *MyoD* is a master switch gene for myogenesis while peroxisome proliferating activating receptor-gamma (PPARγ), C/EBPα and phosphorylated C/EBPβ are involved in adipogenesis (Park et al., 2004; Prusty et al., 2002; Rosen et al., 2002; Ross et al., 2000; Tang et al., 2005). More recently, by overexpressing *C/EBPβ* in pancreatic exocrine derived cells, the formation of hepatic-like cell types has been detected thereby suggesting the importance of C/EBPβ during the conversion of pancreatice cells into liver cells. Furthermore, the potential capacity of pancreatic specific transcription factors including Pdx1 and Ngn3 for the conversion of hepatocytes into pancreatic-like cells has also been investigated (Shen et al., 2003).

1.B.3.1 Neurons derived from retinal pigmented epithelium (RPE)

One demonstration of transdifferentiation was the production of neural retina from

RPE in the newt, chick, rat and human (Dutt et al., 1993; Eguchi and Kodama, 1993; Zhao et al., 1995). It has also been documented that neural retina cells can reversibly convert into RPE in chick embryo (Opas et al., 2001). The molecular basis underlying the transdifferentiation of RPE into neurons was investigated recently. Laminin was the first factor to be identified (Reh et al., 1987). Furthermore, ectopic expression of proto-oncogene H-ras (Dutt et al., 1993), mutation of bHLH transcription factor Mitf (Mochii et al., 1998) and overexpression of activated MAPK/ERK kinase MEK1 (Galy et al., 2002) each contribute to the transdifferentiation of RPE to neuron cells.

1.B.3.2 MyoD - a myogenic determining factor

The role of MyoD in transdifferentiation was found by a rather unusual experimental approach. It had been known that treatment of the mouse fibroblast cell line 3T3 and C3H/10T1/2CL8 with the hypomethylating agent 5-azacytidine is sufficient to produce three different mesenchymal lineages – myocyte, chondrocyte or adipocyte clones (Taylor and Jones, 1979). All three phenotypes persist after withdrawal of 5-azacytidine, suggesting a stable switch has occurred. To identify which gene(s) was responsible for the switch from a fibroblasts to a muscle phenotype, subtracted cDNA probes were generated by isolating cDNA clones present in the 5-azacytidine-treated 10T1/2 cells and the mouse myogenic cell line C2C12 but not in the undifferentiated 10T1/2 cells. One cDNA was identified which, when transfected into 10T1/2 cells, induced stable myoblast expression (Davis et al., 1987). The cDNA was called *MyoD* (Tapscott et al., 1988). It is now known that at least four myogenic regulatory transcription factors (MRFs) are important for skeletal muscle commitment and myotube formation - Myf5, MyoD, myogenin and MRF4 - and all are bHLH nuclear proteins (Parker et al., 2003). To determine whether *MyoD* was indeed the master switch gene for muscle, the transcription factor was introduced into primary

fibroblasts, adipocytes, smooth muscle cells, baby hamster kidney cells, and hepatocytes. Upon *MyoD* expression, the cells started to adopt the muscle phenotype (myotube formation and fusion) and muscle cell markers such as myosin heavy chain (MHC) and myosin light chain (MLC2) were expressed (Weintraub et al., 1989). These results suggest that *MyoD* is indeed the master switch gene for muscle. MyoD can successfully inter-convert cells originating from different germ layers i.e. from hepatocyte (endoderm origin) into a myogenic phenotype (mesoderm origin), however, the muscle differentiation programme was not activated in all cells forced to express MyoD. Expression of MyoD in CV1 (an African green monkey kidney-derived cell) or HeLa (human cervical carcinoma) cells failed to activate differentiated muscle markers (Weintraub et al., 1989).

1.B.3.3 Transdifferentiation into adipocytes

Myoblasts and adipocytes arise from the same germ layer of the embryo, the mesoderm, and recent observations suggest it is possible to directly induce the conversion of myoblasts to adipocytes using G8 myoblast as a model (Hu et al., 1995). The transcription factors C/EBPα and PPARγ, when expressed in G8 myoblasts, can suppress muscle-specific transcription factors (Myf5, MyoD, myogenin and MRF4) and, conversely, upregulate markers specific for adipocytes including aP-2, adipsin, lipoprotein lipase (LPL) and phosphoenolpyruvate carboxykinase (PEPCK). There are also cases where expression of C/EBPα in fibroblast cell lines (such as NIH3T3) will promote adipogenesis (Freytag et al., 1994). It has also been shown that the canonical Wnt signalling pathway is involved in inhibiting preadipocyte differentiation, probably through modulating expression of PPARγ and C/EBPα (Ross et al., 2000). More recently, the transdifferentiation of mouse osteoblastic MC3T3-E1 cells into mature adipocytes by the ectopic expression of PPARγ or PPARγ+C/EBPα was

performed (Kim et al., 2005) and it was shown that PPARγ is a key regulator for the transdifferentiation to adipocytes in this model. Understanding the regulation of C/EBPs and PPARγ is important not only for maintaining the adipocyte differentiation state but also in developing potential therapies for obesity.

1.B.4 Liver and pancreas transdifferentiation

One of the best-documented examples of transdifferentiation is the switch between liver and pancreas, a conversion that reflects the close developmental relationship between the two tissues (Wells and Melton, 1999). Since the liver and pancreas arise from adjacent regions of the embryo during development (mentioned in section 1A), they may express a common transcription factor profile during the early stages of development. Transdifferentiation of pancreas and liver is observed following the exposure of animals to certain carcinogens, or in humans as preneoplastic alterations (Shen et al., 2003; Slack, 1985). The known molecular events underlying the switch between liver and pancreas and other endodermally-derived tissues are discussed below.

1.B.4.1 *In vivo* pancreas to liver transdifferentiation

Ectopic hepatic cells can be generated in the pancreas under a variety of conditions (Grompe, 2003; Shen et al., 2003). Rao et al observed the appearance of multiple foci of hepatocytes in the pancreas after the treatment of rats with a copper depletion-repletion scheme (Rao et al., 1986). The evidence for the presence of hepatocytes in the pancreas was based on the expression of albumin and catalase proteins although the molecular mechanisms mediating the switch in cell phenotype in this model remain elusive. Albumin and α-fetoprotein-positive cells have also been

induced in the islets of Langerhans in transgenic mice expressing *keratinocyte growth factor* (*KGF*) under the control of the insulin promoter (Krakowski et al., 1999). By injection of certain carcinogens such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) into hamsters, the pancreatic hepatocytes were induced and identified by the detection of Periodic Acid Schiff (PAS) staining. The ectopic hepatocytes in the hamster pancreas are morphologically identical to the normal liver (Rao et al., 1988). Additionally, two cases of hepatoid carcinoma arising from malignant islet tumor and pancreatic ductal carcinoma were reported in human patients (Paner et al., 2000).

1.B.4.2 *In vitro* transdifferentiation of pancreatic cells to hepatocytes

More recently an *in vitro* model exhibiting the formation of transdifferentiated hepatocytes in a subclone of the pancreatic-derived cell line AR42J (the subclone AR42J-B13 is referred as B13) has been described and may provide some clues to the mechanisms underlying the changes observed *in vivo* (Burke et al., 2006; Kurash et al., 2004; Shen et al., 2000; Tosh et al., 2002a; Tosh et al., 2002b). Dexamethasone induce hepatic phenotype and C/EBPβ. It was demonstrated that C/EBPβ is important for the transdifferentiation of pancreatic B13 cells to hepatocytes (Shen et al., 2000). Overexpression of *C/EBPβ* induced the loss of the pancreatic phenotype and gain of the hepatic phenotype. The essential requirement for *C/EBPβ* in the transdifferentiation was shown by a dominant negative approach. Introduction of *liver inhibitory protein* (*LIP*), an alternative posttranscriptional product of *C/EBPβ* mRNA, prior to the treatment of B13 cells with dexamethasone, inhibited the induction of transdifferentiation (Shen et al., 2000). Transdifferentiated hepatocytes display a wide range of liver properties including albumin secretion, insulin stimulated lipid deposition, ciprofibrate enhanced catalase expression and induction of acute phase proteins such

as alpha2-macroglobulin (MG) and haptoglobin (Hp) (Burke et al., 2006; Kurash et al., 2004).

1.B.4.3 Conversion of hepatic cells to pancreatic-like cells

Although experimentally less well-documented, recent progress in our understanding of the molecular biology of pancreatic development has taken us several steps closer to elucidating the mechanisms involved in the switch from hepatic to pancreatic cell types. Studies using mouse knockouts have demonstrated that certain transcription factors are essential for the steps in β cell formation: first the specification of a pancreatic rudiment in the endodermal epithelium, then the formation of endocrine precursor cells and finally the commitment to the β cell phenotype (Murtaugh and Melton, 2003; St-Onge et al., 1999; Wilson et al., 2003). In the mouse embryo, a single inductive signal, Fgf, distinguishes the region of endoderm that becomes the liver bud from that destined to become the ventral pancreatic bud (Deutsch et al., 2001), suggesting that only one or a few transcription factors need to have their activity switched on or off in order to interconvert the two tissues. Candidate transcription factors acting as master switch genes to determine a pancreatic fate include *Pdx1*, *Ngn3* or *Neuro D*.

The notion that ectopic expression of *Pdx1* may potentially induce pancreatic-like cells in liver was first demonstrated by Ferber et al using a first-generation adenoviral vector containing *Pdx1* to infect streptozotocin (STZ)-induced diabetic mice (Ferber et al., 2000). The results showed that pancreatic genes were induced and the levels of blood glucose were returned to normal levels (Ber et al., 2003; Ferber et al., 2000). Koizumi et al. also demonstrated the induction of insulin-producing cells in STZ-treated mice after infection with *Pdx1* in a replication-deficient recombinant adenoviral vector (Koizumi et al., 2004). Moreover, Miyatsuka et al revealed that

pancreatic endocrine and exocrine products as well as some functional markers (e.g. islet-type glucokinase and the sulfonylurea receptor) were detected in the liver where *Pdx1* was ectopically expressed in a transgenic mouse model (Miyatsuka et al., 2003). More recently, Cao et al. showed that high glucose culture conditions promotes the maturity of insulin-positive cells derived from liver cells overexpressing *Pdx1Vp16* (Cao et al., 2004). Imai et al. detected the insulin production in the murine liver but found that these insulin producing cells still possessed a hepatic phenotype as indicated by the presence of the liver proteins albumin and transferrin (Imai et al., 2005). All these studies showed the activation of pancreatic genes (e.g. insulin) in liver cells, however, they did not show the formation of differentiated pancreatic cell types.

In previous experiments from our lab using transgenic *Xenopus*, we found that the unmodified *Pdx1* or *Xlhbox8* (*Xenopus* homologue of Pdx1) were not able to bring about transdifferentiation of liver to pancreas. However, inclusion of the *Herpes simplex* protein Vp16 domain in the construct was able to confer transdifferentiation potential with both the mouse and *Xenopus* genes(Horb et al., 2003). The conversion was observed by the presence of GFP in the transdifferentiated pancreas. The GFP was controlled by the pancreatic specific elastase promoter and was expressed in the liver region after ectopic expression of Xlhbox8Vp16 by the liver specific transthyretin (TTR) promoter. A working model is shown in Figure 1.3. The ectopic pancreas in *Xenopus* tadpoles was clearly differentiated, but there are questions about the degree of maturity of the liver at the time of transgene expression (Horb et al., 2003)

Ngn3 is expressed in all endocrine precursor cells and thought to be responsible for the commitment of endocrine cells rather than all pancreatic cell lineages (see section 1.A.3). The role in endoderm development supports Ngn3 as a potential candidate, probably being along with other pancreatic transcriptions factor(s), for the

conversion of liver into pancreas. Only one investigation has shown the induction of the pancreatic gene insulin from liver cells by the expression of Ngn3, or Neuro D, in the presence of Pdx1Vp16 fusion protein (Kaneto et al., 2005). Furthermore, Ngn3 is able to drive pancreatic duct cells to a neuroendocrine phenotype with the formation of insulin-positive cells (Heremans et al., 2002). In addition, using helper-dependent adenovirus (HDAD) encoding *Neuro D*, in combination with betacellulin, the induction of islet neogenesis in the liver was observed and thereby rescue of a diabetic phenotype in mice (Kojima et al., 2003).

(A)
Elastase promoter
GFP
Eggs
L
P
(B)
TTR promoter
XIhbox8
Vp16
Elastase promoter
GFP
Eggs
Transgenesis
In the liver of transgenic tadpoles
XIhbox8Vp16
XIhbox8Vp16
XIhbox8Vp16
XIhbox8Vp16
XIhbox8Vp16
TTR promoter
XIhbox8
Vp16
Transgene expression in liver
TTR promoter is active
XIhbox8Vp16 is expressed in liver cells
Transdifferentiation
Elastase promoter
GFP
Elastase promoter is activated
Partial or complete ectopic pancreas is observed in liver
Pancreas in the liver
EP
P
EP
P
EP
P
EP
P

Figure 1.3 Working model for the transdifferentiation of liver to pancreas in Xenopus tadpoles. (A) Green fluorescent protein (GFP) under the control of the pancreas-specific elastase promoter (Elas) is expressed in the pancreas of transgenic tadpoles. P: pancreas; L: liver. (B) Proposed scheme for transdifferentiation in transgenic Xenopus. Xlhbox8Vp16 is expressed in the liver of TTR-Xlhbox8Vp16; Elas-GFP transgenic tadpoles under the control of the hepatic-specific transthyretin (TTR) promoter. The ectopic pancreas (EP) in the liver is visualized by GFP. Persistent expression of GFP in older tadpoles indicates the stability of the transdifferentiated pancreas. Reproduced from Li et al, 2005

1.B.5 Other metaplasias between internal organs

In addition to the derivation of pancreas from liver, several pieces of evidence have shown that pancreatic-like cells can be generated by the introduction of a single factor. For example, it has been demonstrated that Ngn3 might also be a mediator for converting intestinal epithelial cells to insulin-positive cells following treatment with glucagon-like peptide 1 *in vitro* and *in vivo* (Suzuki et al., 2003). Kawaguchi et al. demonstrated conversion of pancreatic progenitors into duodenal epithelium through the inactivation of PTF1-p48, thereby suggesting a close developmental relationship between intestine and pancreas (similar to that between liver and pancreas) (Kawaguchi et al., 2002). Also, it provides a possible route for creating new pancreatic cells following the overexpression of PTF1-p48 in other cell types such as intestine.

The generation of pancreas from different endodermal organs is not the only case of the transdifferentiation between two internal tissues (Slack, 1985). The appearance of intestinal cells in lung has been shown recently by the expression of hyperactive Wnt signalling (Okubo and Hogan, 2004). This observation was based on the drastically reduced number of Clara cells, a lack of fully differentiated alveolar type 1 and 2 cells and abnormally high proliferation of the bronchial epithelium and the induction of multiple genes (*trefoil factor* 3, defensin related cryptidins 5, 6 and 15), characteristic of intestinal cell types such as Paneth and goblet cells, within the pulmonary epithelium of transgenic lungs. Furthermore, lungs from transgenic mice showed induction or upregulation in the expression of a number of transcription factors that are components or targets of the Notch signalling pathway. These factors include Atoh 1 (Math1), not normally expressed in the lung but active in progenitor cells of the intestine (Yang et al., 2001). The other case is based on the likely function of the homeodomain transcription factor Cdx2 in controlling intestinal rather than

foregut development. Normally Cdx2 is expressed in the caudal part of the endoderm. The induction of gastric intestinal metaplasia was found in transgenic mice in which expression of *Cdx2* was controlled by cis-regulatory elements of the Foxa3 promoter (Silberg et al., 2002). Mutoh et al used the H^+/K^+ ATPase β-subunit gene promoter in a transgenic mouse to drive gastric *Cdx2* expression and again demonstrated intestinal metaplasia (Mutoh et al., 2002). Evidence for the master switch function of Cdx2 is further supported by loss of function in the intestine leading to transformations to squamous epithelium resembling the oesophagus and forestomach (Chawengsaksophak et al., 1997).

1.B.6 Summary

Although transdifferentiation is rare as a natural phenomenon, this does not mean that it will be difficult or impossible to achieve by putting new genes or gene products into cells. With our present understanding of the transcription factors required for specification of various tissues, there should be many opportunities for successful transdifferentiation caused by gene over-expression using either *in vitro* or *in vivo* models.

1.C Diabetes mellitus – onset, classification, complications and treatments

In man, blood glucose is normally maintained at around 4~7 mM before diet and not exceed up to 10 mM after meals. The concentration of circulating blood glucose rises after food intake but is brought back to normal levels by the co-ordinated secretion of the hormones insulin (secreted from the β cells) and glucagon (secreted from the α cells) that reside in the pancreatic islets. After food intake insulin secretion is increased and glucagon secretion reduced. In order to maintain physiological blood glucose values, the pancreatic β cells respond through a series of reactions. These reactions include: (1) sensing the change in blood glucose levels (2) uptake of glucose into the β cells; (3) glucose being metabolised by the glycolytic pathway; (4) depolarisation and calcium entry; and (5) release of insulin granules and further insulin synthesis.

Diabetes mellitus (hereafter simply referred to as diabetes) is a complex metabolic disorder characterised by a relative or absolute lack of insulin. The reasons for this relative or absolute lack of insulin are due to either destruction of the β cells (e.g. by autoimmune antibodies to a glutamic acid decarboxylase GAD65) (Mackay and Rowley, 2004) ; or to a loss of ability of β cells to cope with demand (β cell dysfunction); or to the lack of response to insulin from peripheral organs (insulin resistance). The loss of β cells by autoimmune destruction is referred to as Type I or insulin-dependent diabetes mellitus and the others as Type 2 or non-insulin dependent diabetes. In fact many type 2 diabetic patients are treated with insulin so the term "non-insulin dependent" is something of a misnomer.

1.C.1 Response of β cells to the change of blood glucose

1.C.1.1 Glucose sensing by pancreatic islets

After food intake, carbohydrates are broken down into mono- and disaccharides by the digestive enzymes secreted from the pancreas and intestine. The glucose is absorbed across the intestinal epithelium with the result that the blood glucose level rises. Insulin is released from the pancreatic β cells in response to this physiological challenge resulting in an increase of glucose uptake in peripheral tissues such as skeletal muscle and adipocytes. The insulin stimulated glucose uptake is mediated by GLUT4, a member of the GLUT family of glucose transporters (Foster and Klip, 2000). Once inside the cell, glucose is converted into glycogen in muscle or converted to lipids in adipose tissues (lipogenesis) (Nonogaki, 2000).

Glucose sensing is the first step leading up to the release of insulin from the β cells. In this step, both the high-Km glucose transporter GLUT2 and the high Km-glucose phosphorylating enzyme glucokinase (GCK) are important (Efrat et al., 1994).

In order to respond to an elevated glucose concentration, glucose must enter into β cells. Glucose entry occurs by facilitated diffusion via GLUT2. *GLUT2* was originally cloned from a rat liver cDNA library and is expressed specifically in liver and pancreatic islets (Unger, 1991). A parallel correlation has been shown between reduced GLUT2 levels and loss of glucose-induced insulin secretion in Zucker diabetic mice (Johnson et al., 1990). In addition, hyperglycaemia is observed in transgenic mice overexpressing *GLUT2* antisense RNA. This reveals that GLUT2 might be not only responsible for the transportation of glucose into cells but also

essential for maintaining the glucose homeostasis. However, different observations were presented in other animal models. For example, In RIP-*Hras* transgenic mice (Hras protein is specifically expressed in insulin-producing cells), there is a reduction of GLUT2 but this change did not affect glucose homeostasis (Tal et al., 1992). Furthermore, in man, another member of the glucose transporter family, GLUT1, is more abundantly expressed than GLUT2 in pancreatic β cells (De Vos et al., 1995). These results suggested that the function of GLUT2 may be primarily for the transport of glucose into cells (Schuit, 1997).

GCK is a member of the hexokinase family of enzymes and expressed in pancreatic β cells and hepatocytes. GCK catalyses the ATP-dependent phosphorylation of glucose after glucose entry into pancreatic β cells (Weir et al., 1997) generating glucose-6-phosphate (G-6-P) (Tirone and Brunicardi, 2001). It functions as a glucose sensor in β cells by controlling the rate of entry of glucose into glycolysis and the tricarboxylic acid (TCA) cycle (Matschinsky et al., 1998). A decrease in β-cell glucokinase activity is observed in several situations associated with decreased insulin release. For example, the rat insulinoma RIN-m5F cells secrets little or no insulin in response to glucose and is correspondingly deficient in glucokinase (Meglasson and Matschinsky, 1986). It was also reported that the impaired GCK activity is related to one type of an autosomal dominant mode of inheritance mature-onset diabetes of the young, MODY 2 (Froguel et al., 1993). This further confirms the importance of GCK for insulin secretion and glucose homeostasis.

1.C.1.2 Insulin secretion

Metabolism of glucose through the glycolytic pathway yields two molecules of ATP (MacDonald et al., 2005). As a result of the increase in ATP production, the

intracellular ATP/ADP ratio is increased. This change leads to closure of K_{ATP} channels which then results in depolarisation of the cell membrane. Ca^{2+} accumulates in the membrane-depolarised cells via activation of the VGCC or release of Ca^{2+} from the ER. In addition to the K_{ATP}-dependent pathway, PKA and protein kinase C (PKC) pathways are activated by sensing the glucose metabolic signalling in a K_{ATP}-independent manner although the molecular basis is not yet known (Bratanova-Tochkova et al., 2002).

Biphasic insulin secretion is observed in the pancreas following rapid and sustained stimulation of β cells with glucose (Henquin et al., 2002). The immediate reaction, so-called first phase insulin release, is mediated by rapid release of the insulin granule pools in a Ca^{2+}- dependent manner (Straub and Sharp, 2004). The insulin granule populations are complex, consisting of three pools including a reserve pool, a docked pool and a readily releasable pool. The increase of the intracellular Ca^{2+} concentration is accelerated not only by the closure of K_{ATP} channel but also by several K_{ATP}-independent mechanisms (Straub et al., 2002). The orchestration of K_{ATP}-dependent and K_{ATP}-independent signals positively augments the pre-existing insulin granules to release insulin. Under prolonged exposure to a high glucose environment, the demand for insulin to maintain normal physiological glucose homeostasis drives the next round of insulin production, namely insulin biosynthesis.

1.C.1.3 Insulin biosynthesis

Insulin is synthesised in response to prolonged elevated blood glucose. Briefly, the sequence of events is as follows: (1) production of preproinsulin transcripts in the cell nucleus; (2) packaging of preproinsulin into proinsulin in the rough endoplasmic reticulum (RER); (3) synthesis of mature insulin from proinsulin in the Golgi apparatus and lastly (4) precipitation of insulin protein in the secretory granules (Figure 1.4).

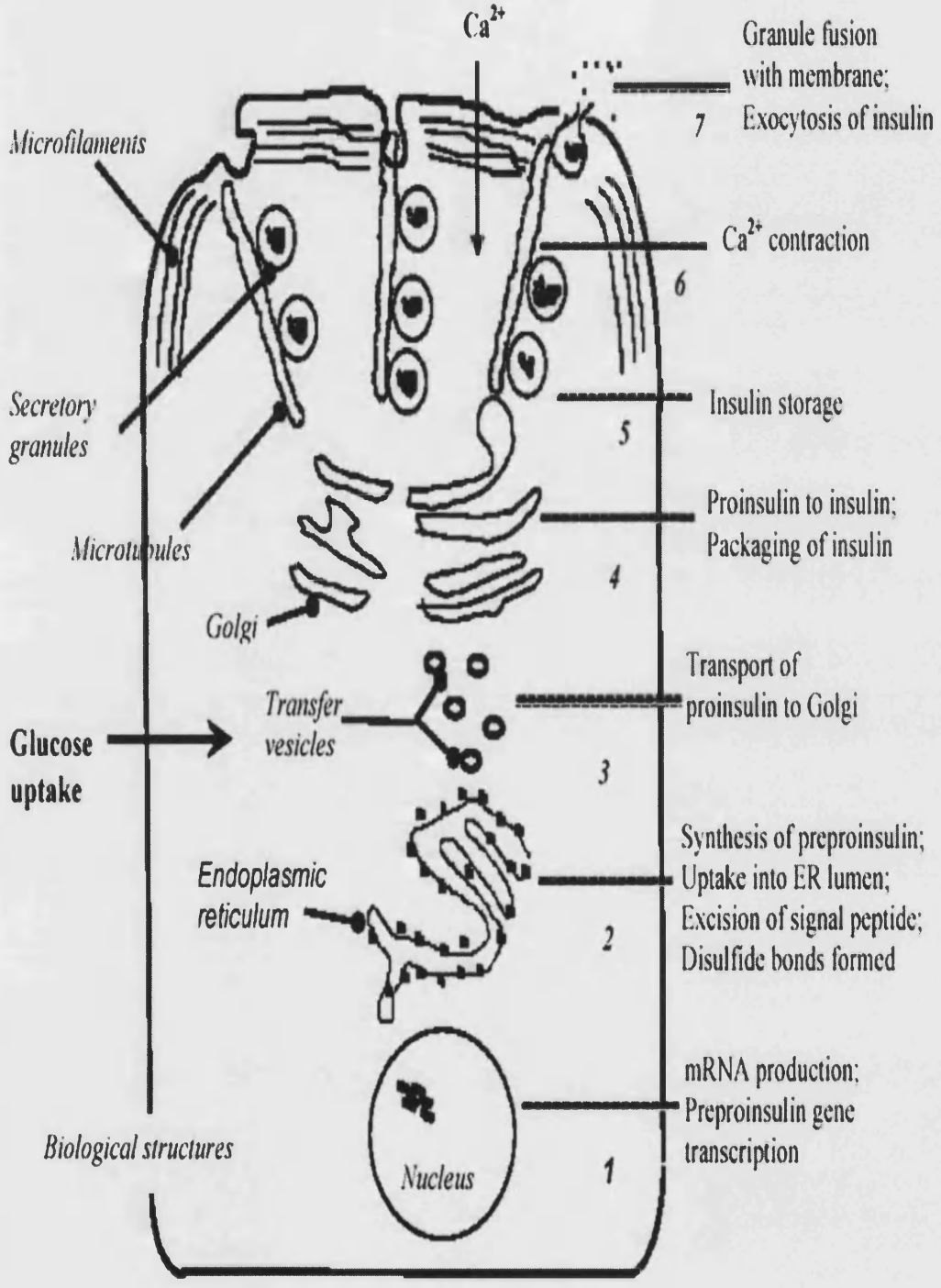


Figure 1.4 Illustration of insulin secretory pathway. Details are discussed in chapter 1.C.1.2 and 1.C.1.3. Reproduced from Tischler 2004

The preproinsulin is the original single polypeptide used to form mature insulin. It consists of four regions, from N-terminal to C-terminal, a hydrophobic signal sequence (SS), B-chain polypeptide, connecting peptide (C-peptide) and A-chain polypeptide (Figure 1.5). Figure 1.6 showed the processing of preproinsulin to folded proinslin. Briefly, the transcribed preproinsulin mRNA moves to a ribosome and sequentially binds to the signal recognition particle (SRP) initiating protein translation. The translated SS peptide (called the nascent chain) tags onto the SRP and migrates to a high-affinity receptor for SRP named the SRP docking protein (SRPdp) that is located at the external surface of RER (Gilmore et al., 1982). Subsequently, the ribosome dissociates from the SRP and SRPdp and is restrained by the ribosome receptor (RR) on the ER membrane. The natural hydrophobic property of SS (22 of 24 amino acids in SS are hydrophobic) facilitates and enhances the binding affinity between ribosome-mRNA complex and the phospholipid bilayer of the ER membrane (Do et al., 1996). In addition to SRPdp, other membrane proteins such as translocating-chain associated membrane (TRAM) and Sec61 proteins are essential for directing the ribosome-mRNA complex to the ER membrane (High et al., 1993; Knight and High, 1998; Voigt et al., 1996). Through the joint interaction of membrane proteins, the complex is placed at the site on the ER membrane termed a translocon (McCormick et al., 2003) where the SS peptide guides the extension of the preproinsulin polypeptide chain into the ER lumen. The SS peptide is excised by signal peptidase positioned on inner ER membrane and proinsulin is formed. The native proinsulin is then folded by a molecular chaperone (e.g. protein-disulfide isomerase, PDI) by the formation of the disulfide bonds (Winter et al., 2002).

The vesicles filled with proinsulin then bud out from the RER towards the Golgi apparatus where the zinc / calcium rich environment around the Golgi favours the formation of the zinc-containing proinsulin hexamer (Dodson and Steiner, 1998).

Proinsulin passes through *cis*, *medial*, and *trans* Golgi cisternae and is converted to insulin at the stage of vesicle formation. This transformation is driven by membrane-associated prohormone convertase enzymes (PCs). The catalytic sites targeted by PCs are called the dibasic sites (Goodge and Hutton, 2000). Subsequently, the granules are packed with mature insulin, coupled with the precipitation of removed C-peptide and are maintained in the cytoplasm as an "insulin secreting vesicle pool". Once the β cells sense certain physiological challenges such as high glucose, the insulin is secreted through the release of these primary granules in order to maintain normoglycaemia.

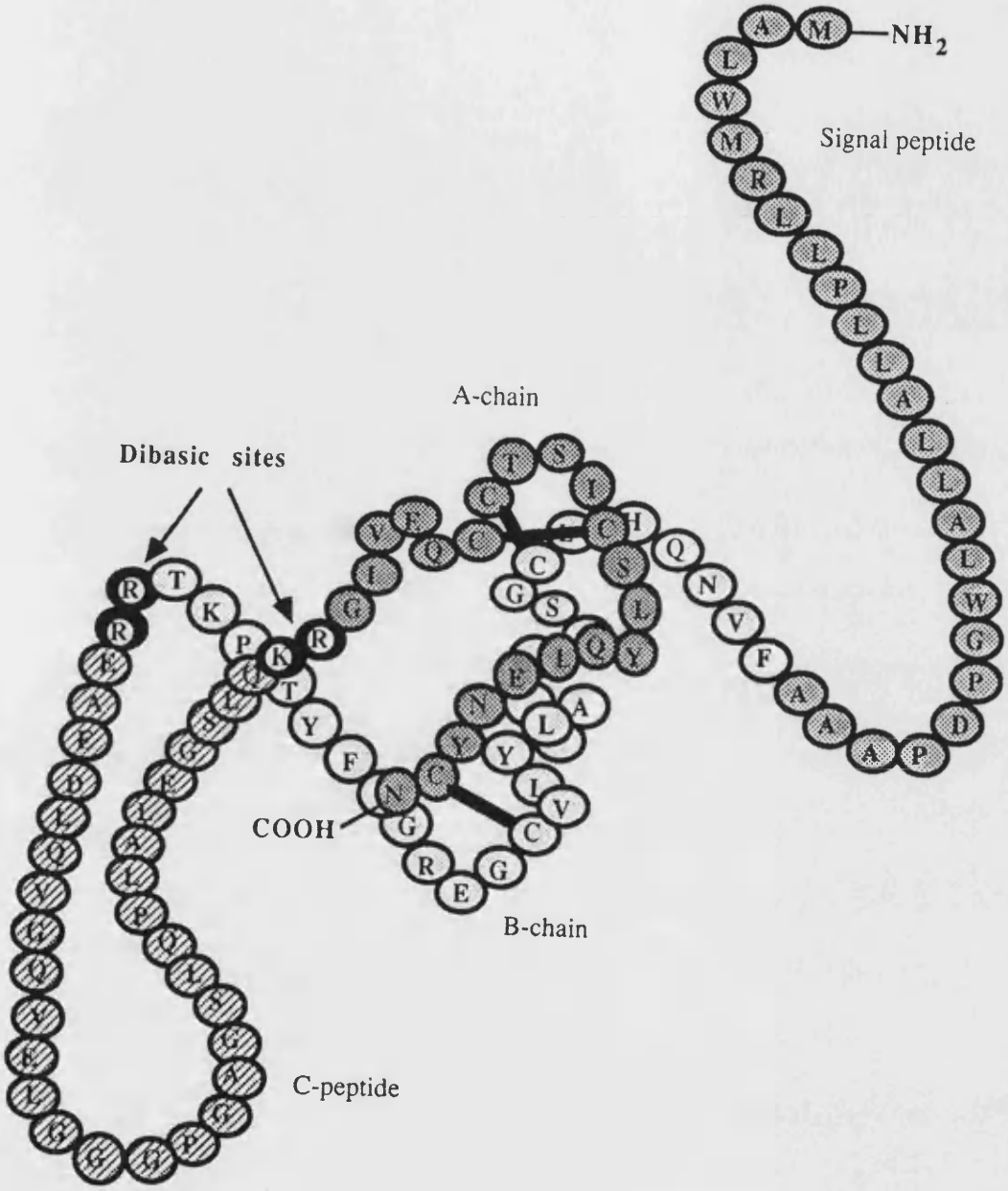


Figure 1.5 The amino acid sequence of human preproinsulin. The four domains of preproinsulin are shown. See chapter 1.C.1.3 for details. Reproduced from p.65 of Insulin: Molecular Biology to Pathology, 2nd edition, Edited by F.M. Aschroft and S.J.H. Aschroft, 1992

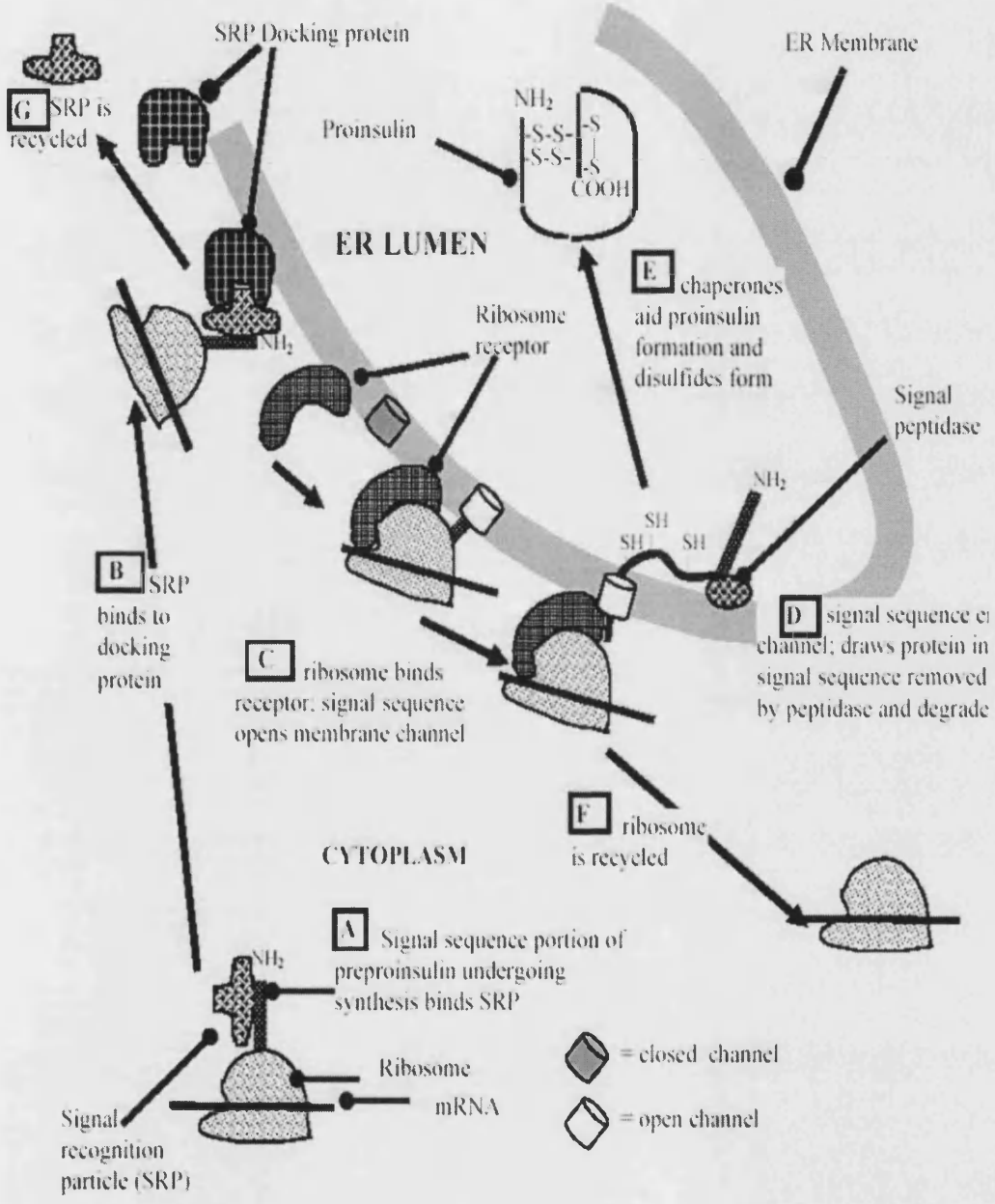


Figure 1.6 Mechanism for uptake of preproinsulin into the lumen of the ER and subsequent processing to proinsulin. Details are described in chapter 1.C.1.3. Reproduced from Tischler 2004

1.C.2 Classification of Diabetes mellitus

A relative or absolute lack of insulin means that glucose cannot enter insulin-responsive cells (i.e. muscle and adipocytes). Under these circumstances, diabetes results. Diabetes is the name given to the clinical description of patients with a number of symptoms arising from raised glucose levels. A number of pathological complications arise in diabetic patients if the blood glucose levels are poorly controlled. Examples of complications include angiogenic disorders (cardiovascular disease, hypertension, retinopathy), neuropathy and kidney dysfunction. One of the possible molecular mechanisms responsible for these complications is via the increased oxidative stress induced by chronic hyperglycaemia (King and Loeken, 2004). In brief, persistent hyperglycaemia will drive insulin-independent tissues to take up glucose. The increased glucose will enter glycolysis, Krebs cycle and oxidative phosphorylation pathways (King and Loeken, 2004). This increased flux generates excess reactive oxygen species (ROS) (see Figure 1.7) and the accumulated ROS elicits the increased oxidative stress and may interfere with normal function of tissues. For example, the ROS increase: blood flow in the retina (Kowluru and Kennedy, 2001) (retinopathy) and contractility of vascular smooth muscle cells (Sharpe et al., 1998) (vascular disorders). ROS decreases neural conductivity in peripheral nerves (Hounsom et al., 2001) (neuropathy). Another major factor in the development of the diabetic complications may be the increased glycosylation of proteins. This can be lead to the accumulation of advanced glycosylation end-products and lipoxidation end-products (AGEs/ALEs) (Wada and Yagihashi, 2005). AGEs/ALEs can induce cross-linking of proteins or induce specific cellular responses including the release of profibrogenic and proinflammatory cytokines thus causing diabetic retinopathy and nephropathies such as glomerulosclerosis,

interstitial fibrosis, and tubular atrophy (Bohlender et al., 2005). In addition to ROS and AGEs/ALEs, VEGF in diabetic retinopathy (Aiello, 2005) and microalbuminuria for cardiovascular and renal disorders in T2DM (Lane, 2004) has also been shown.

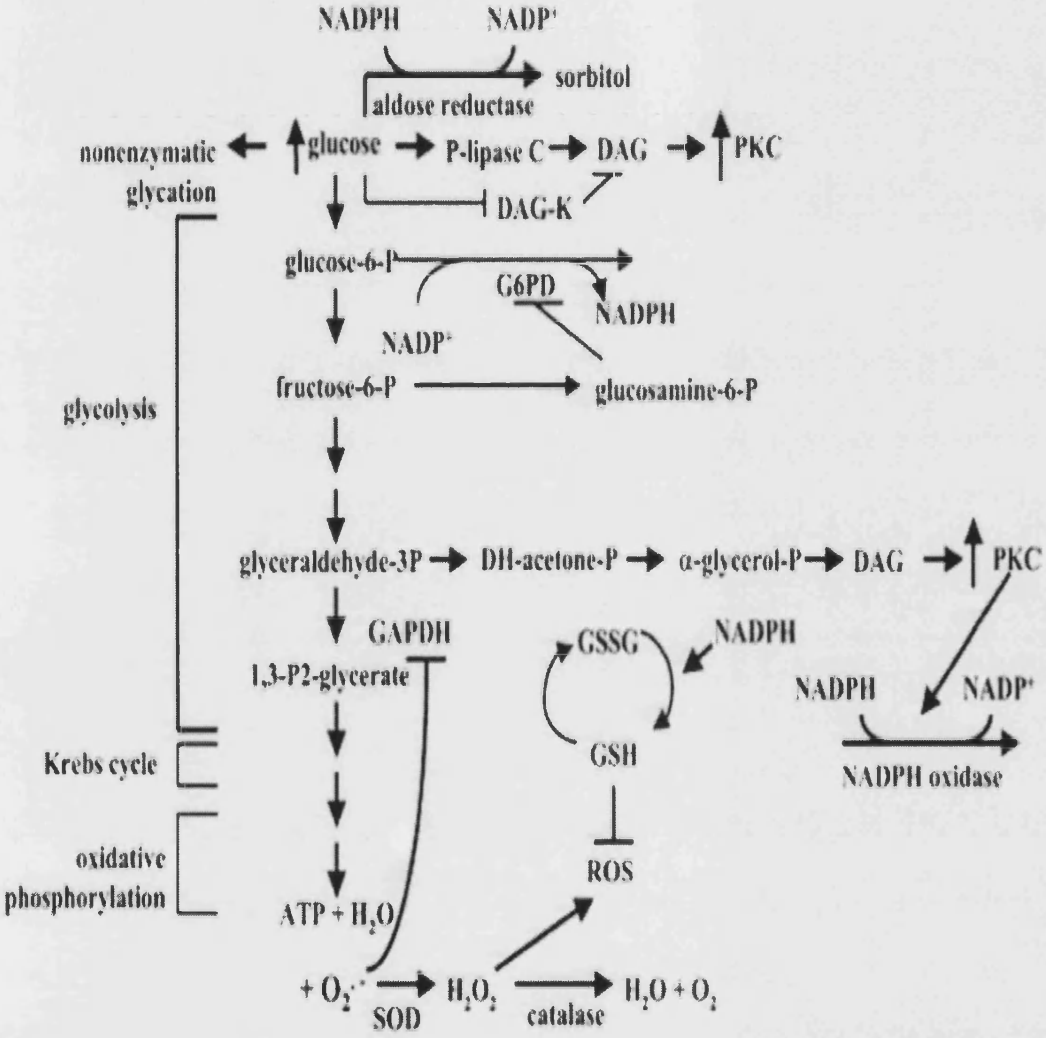


Figure 1.7 Schematic diagram of pathways that contribute to oxidative stress in response to increase glucose flux. Reproduced from King and Loeken 2004.

1.C.2.1 Type 1 diabetes mellitus (T1DM)

As previously mentioned, there are two types of diabetes T1DM and T2DM. T1DM is much less common than type II diabetes and the patients are

insulin-dependent. Type I diabetes usually appears suddenly in non-obese children or young adults. This is why it has been referred to as juvenile-onset diabetes. T1DM is an autoimmune disease characterised by the destruction of the β-cells. This occurs as a result of direct attack by aberrant β-specific autoimmune antibodies (Narendran et al., 2005). T1DM is characterised by the presence of antibodies and T-cells response to islet proteins. Histological analysis of islets shows lymphocyte attack which is not present in a type 2 or a healthy individual (Foulis et al., 1987). Further evidence to suggest an autoimmune disease comes from experiments performed using immunosuppressor to modulate T1DM. Immunosuppressive drugs such as cyclosporin and azathioprine can slow the progression of β cell destruction and have been used in prevention trials for T1DM (Narendran et al., 2005). Candidates for the islet auto-antigens include islet cell antigen (Narendran et al., 2005), insulin or proinsulin (Palmer et al., 1983), glutamic acid decarboxylase (GAD) (Baekkeskov et al., 1990) and protein tyrosine phosphatase (IA-2) (Narendran et al., 2005). Interestingly, the diabetic phenotype (i.e. glucose intolerance) is not detected until the late stage of the disease in spite of the presence of autoimmune antibodies for several years (Knip et al., 1994). This observation suggested that the preclinical period of T1DM is long and might allow the provision of appropriate treatment so as to save any residual β cell mass or even prevent T1DM (Narendran et al., 2005).

1.C.2.2 Type 2 diabetes mellitus (T2DM)

T2DM is a complex illness with both genetic and environmental pathogenic elements. T2DM patients are usually obese, have disturbed insulin secretion and peripheral insulin insensitivity (Beck-Nielsen and Groop, 1994). The onset of T2DM is due to the prolonged exposure of pancreatic β cells to glucose. This eventually leads to β-cell exhaustion and glucose toxicity. The effect of β-cell exhaustion is the early

form of the disease and is likely to be reversible whereas the glucose toxicity (or called β-cell failure) occurs later in the disease and is less reversible (Rossetti et al., 1990). More recently, a harmful effect of elevated levels of free fatty acids on β-cell function have also been studied and suggested that diabetes is not only the carbohydrate disease but is also associated with disordered lipid metabolism (Unger, 1995). In addition, the deposits of islet amyloid present in T2DM diabetic pancreas may lower the functional β-cell mass (Kahn et al., 1999; Marzban et al., 2003). Several lines of evidence indicated that hyperglycaemia induces β-cell apoptosis by inducing apoptotic molecules including glucose-induced NF-κB, apoptotic-associated protein caspase 3 and ROS. Once the apoptosis-related factors have accumulated, β-cell mass is reduced, insulin secretion is impaired and eventually diabetes occurs (Mandrup-Poulsen, 2003). Insulin resistance and β-cell dysfunction (exhaustion of β cells) are the two major clinical characteristics found in T2DM diabetic subjects.

As described in detail in section 1.C.1, insulin acts to maintain euglycaemia and this process involves several steps. A significant disruption of any of these actions regardless of the presence of normal or even high circulating levels of insulin indicates the presence of "insulin resistant" phenotype (Reaven, 1988). Insulin resistance is the first defect to be detected in T2DM and must be accompanied by β cell deficiency for full development of the disease (Martin et al., 1992). The early insulin resistance in insulin-targeted organs including liver, muscle and adipose tissues starts to be detected during pre-diabetic stage and therefore elevated insulin is subsequently produced from β cells to compensate the require of insulin to maintain glucose homeostasis. Over a period of time, however, overexpression of insulin does not match the requirement of insulin to uphold euglycaemia and the next pathological condition, β-cell failure, occurs. Furthermore, the expansion of β-cell mass, namely islet hyperplasia, is another notable and consistent feature associated with insulin

resistance in T2DM animals (Tokuyama et al., 1995).

The working model for the β-cell dysfunction in T2DM can be discussed using a T2DM animal model, 90% pancreatectomy (Px) rats. It was found that insulin secretion is enhanced (hypersensitivity) during the period when the blood glucose is as stable as the level in control animals (within 2 weeks post surgery) in Px rat (Bonner-Weir et al., 1983). This can be explained by the idea that, in order to compensate for the demands of insulin, β cells overwork to produce sufficient insulin to reach normoglycaemia. Another effect that accompanies the hypersecretion of insulin from β cells in Px rats is the increase of glucokinase activity. This increased activity is thought to increase glucose sensitivity (discussed in section 1.C.1.1). Additionally, an increase in the low-Km hexokinase activity is also observed in 90% of diabetic rats in comparison with the non-diabetic control animals (Hosokawa et al., 1995). Higher activity of hexokinase results in increased sensitivity of β cells even when the blood glucose concentration is low. Thus, established hyperglycaemia and enhanced β-cell hexokinase activity lead to very high level of insulin output (Cockburn et al., 1997). Since the insulin-targeted tissues are insulin-resistant, the hyperglycaemia is more serious in spite of constitutive insulin secretion. Finally, overworked β cells become fatigued and insulin granules are depleted.

The occurrence of dysfunctional insulin receptor (IR) and downstream kinase provided another mechanism to explain why insulin resistance occurs. In the peripheral organ (e,g, muscle and liver) of insulin resistant subjects, a defect of IR and the insulin receptor kinase was observed (Farese et al., 2005; Kellerer and Haring, 1995). Defects in IR function were originally determined by the detection of IR-specific autoimmune antibody, the mutation on IR and the decreased IR numbers (Kahn et al., 1996; Taylor et al., 1990). However, this defect is unlikely to be significantly contributing to the pathology of diabetes while the antibody titre is generally low and

large changes of insulin-IR binding have never been seen (Kahn et al., 1996). More attempts therefore were turned to investigate the effect of deficient IR kinase for the onset of insulin resistance. Insulin-IR binding activates insulin receptor substrate-1 (IRS-1)-dependent phosphatidylinositol 3 - kinase (PI3K) which activates atypical protein kinase C (aPKC, namely PKCλ and ζ) and protein kinase B (PKB/Akt) pathways and thereby regulates glucose transport (see Figure 1.8) (Schinner et al., 2005). The results exhibiting the reduced Akt kinase activity in the skeletal muscle tissue of T2DM patients (Krook et al., 1998) and defective activation of aPKC in muscles and adipocytes of rats with T2DM (Kanoh et al., 2001) suggested that IR-related kinase plays a major role for the development of insulin resistance.

1.C.2.3 Maturity-onset diabetes of the young (MODY)

MODY is a specialised type of T2DM since it is characteristic of an onset before the age of 25 years and usually in childhood and adolescence (Fajans et al., 2001). MODY can result from the mutation in any one of at least six genes. One of them, MODY2, encodes the glycolytic enzyme glucokinase (Froguel et al., 1993) whereas the other five types of MODY are associated with pancreatic transcription factors: HNF4α (related to MODY1) (Yamagata et al., 1996a), HNF1α (associated with MODY3) (Yamagata et al., 1996b), Pdx1 (associated with MODY4) (Stoffers et al., 1997), HNF-1β (associated with MODY5) (Horikawa et al., 1997) and NeuroD1 (associated with MODY6) (Malecki et al., 1999). These genes are all expressed in pancreatic β cells and the disruption of each gene leads to β-cell dysfunction, and eventually diabetes. In comparison to late-onset T2DM, the onset of MODY is more likely to be a genetic issue. This statement is supported by the fact that the screening of family pedigrees of MODY diabetic patients showing a history of diabetes in three or four generations(Busch and Hegele, 2001; Fajans et al., 2001).

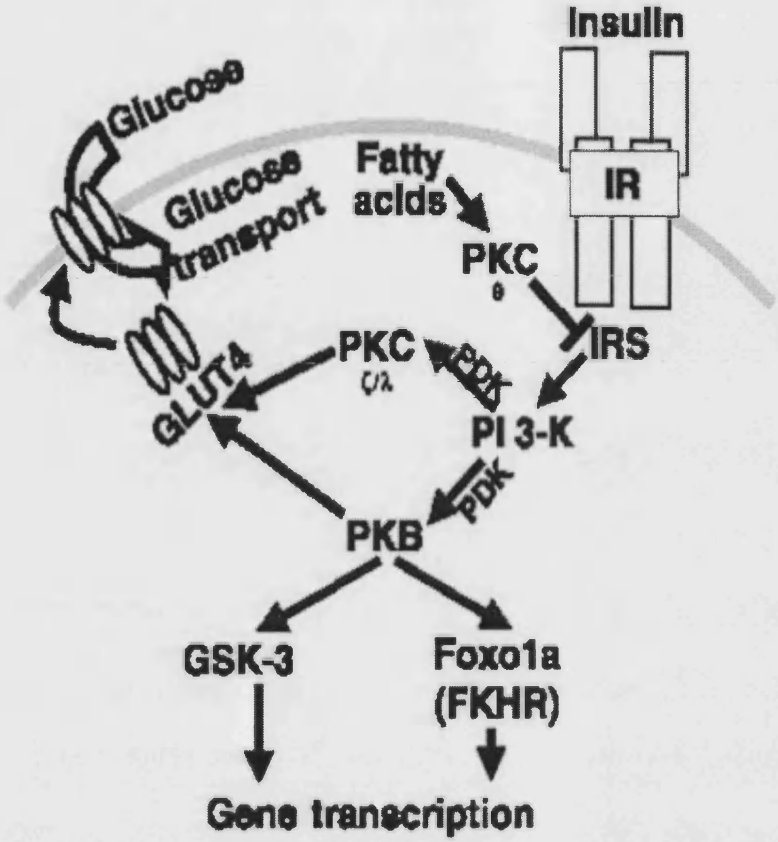


Figure 1.8 Schematic representation of insulin signalling. Binding of insulin to the IR activates PI3-K through IRS. Downstream of PI3-K, the phosphoinositide-dependent kinase (PDK) mediates activation of PKB. Activated PKB can regulate transcription of target genes via glycogen-synthase kinase-3 (GSK-3) or Foxo1a. Also, PKB regulates glucose uptake by recruiting GLUT-4 to the plasma membrane. Downstream of the IR the signalling cascade branches with PKCλ and ζ as additional downstream effectors of insulin. Modulation of PKC θ activity by fatty acids may impair signalling through IRS/ PI3 kinase and GLUT4-dependent glucose transport. Abbreviations are shown in text. Reproduced from Schinner et al 2005

1.C.2.4 Animal models of diabetes mellitus

In order to mimic the diabetic phenotype, several rodent models have been established based on the features of various types of DM (Rees and Alcolado, 2005). For T1DM, toxic agents specific for β cells are usually used. One of the best known is streptozotocin (STZ). STZ is a powerful alkylating agent and activated through the binding of GLUT2. The administration of STZ induces multiple DNA strand breakage

and hence specifically destroys β cells (Szkudelski, 2001). In addition, inbred non-obese diabetic (NOD) mice exhibiting insulitis was also generated as a model for T1DM study (Rees and Alcolado, 2005). T2DM represents a heterogeneous group of disorders, therefore animal models of T2DM are more complex. Obese animals, such as the ob/ob mice, db/db mice and Zucker (fa/fa) rats are used. These animals are all deficient for leptin or leptin receptor and develop insulin resistance thereby quickly developing hyperglycaemia (Chagnon and Bouchard, 1996; Lee et al., 1996).

1.C.3 Treatments for diabetes mellitus

A major aim of glucose-lowering therapy in people with diabetes is to delay or prevent the complications of diabetes that threaten the quality and duration of life. The current treatment for DM can be divided into several categories. One of the basic therapies is insulin administration in DM patients. The primary goal of insulin therapy is to replace endogenous insulin secretion in patients with T1DM or T2DM between meals and overnight as well as postprandially. However, this treatment fails to provide tight blood glucose control, interferes with patients' life quality and possibly can in some cases cause hypoglycaemia(Devries et al., 2004). Recent advances in insulin therapy include the insulin pump technology to make continuous subcutaneous insulin infusion (CSII) (Stephens and Riddle, 2003) and the improvement (i.e. requirement for smaller blood samples and the possibility of continuous measurement) of glucose monitoring systems (Goldstein et al., 2003).

Drugs that increase insulin secretion (insulin secretagogues), or decrease the insulin resistance, have also been used as routine treatments for diabetic patients. Insulin secretagogues may be divided two groups, the initiators and the potentiators. The former are substances that are capable of stimulating insulin release on their own

whereas the insulin potentiators comprise a number of hormones and are able to induce insulin secretion in the presence of glucose only (Rustenbeck et al., 2004). The most commonly used insulin secretagogues are the sulphonylurea drugs. The principal target for sulphonylurea is the ATP-sensitive potassium channel (K_{ATP}) that controls cell membrane potential in pancreatic β cells (Proks et al., 2002). Once the sulphonylurea binds the K_{ATP}, the level of cytosolic Ca^{2+} increases and insulin is released (Proks et al., 2002). In order to reduce insulin resistance, metformin, thiazolidinediones and other hypoglycaemia agents are used (Seufert et al., 2004). Taking metformin as an example, it helps to lower the blood glucose by (i) suppression of hepatic glucose production and (ii) increasing insulin-mediated muscle glucose uptake. Metformin also reduces fatty acid oxidation (Bailey and Turner, 1996). Moreover, metformin also possesses several nonglycaemic effects such as stabilising body weight by preventing weight gain (Bailey, 2005). In addition to the aforementioned medical therapy, regular exercise to control body weight is beneficial for all diabetic patients (Hopkins, 2004). Physical training results in lower fasting and postprandial insulin concentration and increase insulin sensitivity (LaMonte et al., 2005). In patients with T1DM, reduced insulin resistance leads to less requirements of insulin administration and, for T2DM patients, increased insulin sensitivity might result in improved glycaemic control.

The best treatment for DM is whole pancreas or islet transplantation. Transplantation has received much attention since an improved protocol for immunosuppression has been introduced by a group in Edmonton (Shapiro et al., 2000). However, the main limitation for islet transplantation is the shortage of isolated islets from cadavers (Ohgawara et al., 2004). This problem could be overcome by: (1) generating xenotransplantable islets (xenotransplantation: the transplantation of organs/tissues of other species origins); (2) bioengineering of functional islets, or

insulin-producing β cells, from various cell sources including stem cells, tissue specific progenitors and even differentiated cells (transdifferentiation) (Bonner-Weir and Weir, 2005). For example, under certain chemical or genetic manipulations, the generation of pancreatic islet-like cells from embryonic stem cells, pancreatic ductal and acinar cells and liver cells have been proposed from many groups (Katdare et al., 2004; Kim et al., 2003; Lumelsky et al., 2001; Ogata et al., 2004; Sapir et al., 2005; Zulewski et al., 2001). In addition, it has also been demonstrated that human peripheral blood monocytes can be reprogrammed into pancreatic-like β cells by incubating in pancreatic islet-specific differentiation media after the treatment of macrophage colony-stimulating factor and interleukin 3 (Ruhnke et al., 2005).

A different approach to induce β-like cells has been to take intestinal K cells and engineer them by providing the human insulin gene linked to the 5'-regulatory region of the gene encoding glucose-dependent insulinotropic polypeptide (GIP). In mice expressing this transgene, human insulin is specifically produced in gut K cells. To investigate if the expression of insulin in K cells can protect the mice from developing diabetes, STZ was introduced into either GIP transgenic mice or aged control animals to induce diabetic phenotype and the blood glucose level was determined. The results showed that neither glycosurea not fasting hyperglycaemia was observed in GIP transgenic mice whereas STZ resulted in the hyperglycaemia and the presence of glucose in urine in control animals (Cheung et al., 2000). Adult stem cells residing in the ductal region of pancreas are also able to generate functional β cells *in vitro* (Bonner-Weir et al., 2000; Ramiya et al., 2000). Using a cell lineage tracing strategy, a recent study suggested that insulin-secreting cells can be generated from adult pancreatic acinar cells (Minami et al., 2005). Interestingly, an *in-vitro* culture study proposed that β cells can de-differentiate into fibroblast-like cells and then re-differentiate to β cell phenotype (Gershengorn et al., 2004; Lechner et al., 2005).

This study shows the possibility to transform mesenchymal cells into pancreatic β cells. This idea is also supported by an observation that the conversion of the nestin-positive cells (i.e. mesenchymal cells) into insulin-producing cells can be promoted by expressing a pancreatic-specific transcription factor Pax4 (Blyszczuk et al., 2003).

ES cells, in contrast to all the other sources of β cells, are more advantageous for the generation of β cells since they (i) can be derived from patients by somatic cell nuclear transfer (SCNT) technique (Fulka et al., 1998), (ii) have virtually unlimited capacity and (iii) can give rise to all cell types in the body including the differentiated β cells when they are in the right environment (Colman and Kind, 2000; Nir and Dor, 2005). However, spontaneous differentiation of ES cells only produced extremely low number of cells that express β cell markers. The molecular basis to direct from the ES cells into functional pancreatic β cells is also still poorly understood (Nir and Dor, 2005; Rajagopal et al., 2003). Recently, Soria and colleagues successfully induced differentiation of mouse ES cells into pancreatic β cells by a 3-step *in vitro* differentiation method consisting of directed differentiation, cell-lineage selection, and maturation (Soria, 2001). By using these purified β cells, the blood glucose concentration of the streptozotocin-induced diabetic mice was normalised following transplantation of the ES cell-derived β cells. This result therefore opens new possibilities for tissue transplantation in the treatment of diabetes even though the identification of the molecular basis associated with this conversion process is remained to be determined (Leon-Quinto et al., 2004; Vaca et al., 2005).

1.D Aims

The present project had the following aims:

1. True transdifferentiation of liver to pancreas has never convincingly been demonstrated (Ber et al., 2003; Ferber et al., 2000; Imai et al., 2005; Kojima et al., 2003; Miyatsuka et al., 2003; Sapir et al., 2005). Although results of some investigations appear to show the conversion of liver cells into pancreatic-like cells, almost all of the investigations show only the detection of a "mixed-cell" phenotype, namely the liver cells expressing pancreatic markers rather than becoming *bone fida* pancreatic cells. As described in section 1.B.4.3, in an earlier observation from our laboratory, it has been described the appearance of ectopic pancreas in transgenic tadpoles of *Xenopus levies*. It was also shown the expression of amylase and insulin proteins in cultured human hepatoma HepG2 cells (Horb et al, 2003). Therefore, in order to unambiguously determine whether it was possible to induce the true transdifferentiation of hepatic cells to pancreatic cells, changes at both cellular and molecular levels were examined. The human hepatoma cell line HepG2 was used in this study. Briefly, the main aims of this project was to determine: the alteration of pancreatic and hepatic phenotype in transdifferentiated cells; the ancestor-descendant relationship between parental and transdifferentiated cells and finally to characterise the functional nature of the transdifferentiated cells.

2. Due to the limitations of HepG2 cells, it was then decided to investigate the potential of other liver cell models to undergo transdifferentiation to pancreatic cells. The first was based on culture of embryonic liver and the second involves isolation and culture of adult rat hepatocytes. The embryonic liver tissues were cultured following the same technique that has been established in the lab and established a good delivery system for introducing genes to induce transdifferentiation. To isolate

hepatocytes, standard 2-steps perfusion procedure was used. The isolated hepatocytes were then cultured in the chemically-defined medium to maintain the differentiation properties and further to induce the conversion into pancreatic-like cells after the introduction of one or several pancreatic-specific transcription factors.

Chapter 2.
Materials & Methods

2.A Materials

All chemicals were dissolved in distilled water unless otherwise stated.

2.A.1 General chemicals and buffers

Glucagon-like peptide 1 (GLP-1, fragment 7-37), human recombinant betacellulin, EDTA (stock=0.5M), 3-aminopropyltriethoxysilane (APTS), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), phenobarbital, dexamethasone, Oil red O, trypan blue, urease buffered solution, phenol nitroprusside, alkaline hypochlorite, L-arginine, Isonitropropiophenone (ISNP) and $MnCl_2$ were all purchased from Sigma, UK. Tris base, chloroform, 2-propanol, ethanol and acetone were from Fisher Scientifics, UK. PBS powder was obtained from BIOCHROM AG, Berlin, Germany.

2.A.2 Cell culture reagents

The rat pancreatic-exocrine cell line, AR42J-B13 (provided by Dr. Itaru Kojima, Tokyo, Japan) was cultured in Dulbecco's Modified Eagle's Medium containing 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 10% Foetal Bovine Serum. The human hepatoma cell line HepG2 was obtained from the European Collection of Cell Cultures (ECACC, UK) and maintained in Dulbecco's Modified Eagle's Medium (with 5.5mM glucose or 25mM glucose in, Sigma, UK) containing 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 1 X MEM Non-Essential Amino Acids (all were from Sigma, UK) and 10% Foetal Bovine Serum (Gibco™/Invitrogen Life Technologies). The RIN-5F insulinoma cell line was purchased from ECACC and kept in RPMI1640 medium (Sigma, UK) containing 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 10% Foetal Bovine

Serum. Human pancreatic ductal cell line PANC-1 (ECACC), was cultured in Dulbecco's Modified Eagle's Medium containing 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 10% Foetal Bovine Serum. Serum free medium for the culture of isolated rat hepatocytes were Williams' Medium E (Sigma) or KSFM (Gibco™/Invitrogen Life Technologies) containing 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 50 µg/ml gentamycin, 100 ng/ml Fungizone (both from Gibco™/Invitrogen Life Technologies) with indicated supplements (see below).

Table 2.A.1 DNA preparation reagents

Kit	Components	Supplier
QIAEX II gel extraction kit	Buffer QX1 QIAEX II suspension Buffer PE	Qiagen, UK
Wizard Plus SV Minipreps DNA purification system	Cell resuspension solution Cell lysis solution Alkaline protease solution Neutralisation solution Column wash solution Nuclease-free water Mini-spin column	Promega, UK
Wizard Plus SV Maxipreps DNA purification system	Cell resuspension solution Cell lysis solution Alkaline protease solution Neutralisation solution Column wash solution Nuclease-free water Maxi-spin column	Promega, UK

Table 2.A.2 Antisera for immunofluoresence staining

Primary antibody	Species	Dilution factor	Supplier
Amylase	Rabbit	1/300	Sigma, UK
Albumin	Rabbit	1/300	Sigma, UK
α1-antitrypsin	Rabbit	1/100	Sigma, UK
Apolipoprotein B (ApoB)	Mouse	1/100	Chemicon International
Carbamoyl phosphate synthetase I (CPS I)	Rabbit	1/300	a gift from Dr. Wouter H. Lamers, University of Amsterdam, The Netherlands
C/EBPα	Rabbit	1/100	Santa Cruz Biotechnology
C/EBPβ	Mouse	1/100	Santa Cruz Biotechnology
human C-peptide	Rabbit	1/100	Acris Antibodies
Connexin 32	Mouse	1/100	Zymed Laboratories Inc.
Cyp2E1	Rabbit	1/600	a gift from Dr. Magnus Ingelman-Sundberg, Karolinska Institute, Stockholm, Sweden
E-cadherin	Mouse	1/100	BD Transduction Laboratories, USA

β-galactosidase	Rabbit	1/100	MP Biomedicals UK
Glucagon	Mouse	1/100	Sigma, UK
Glutamine synthetase(GS)	Mouse	1/300	BD Transduction Laboratories, USA
Green fluorescence protein(GFP)	Mouse	1/300	Sigma, UK
Haptoglobin	Rabbit	1/200	Sigma, UK
HNF4α	Rabbit	1/100	Santa Cruz Biotechnology
Insulin	Guinea pig	1/300	Sigma, UK
IPF1(Pdx1)	Rabbit	1/100	JMW Slack
Pancreatic polypeptide (PP)	Rabbit	1/100	Zymed Laboratories Inc.
RXRα	Rabbit	1/150	Santa Cruz Biotechnology
Somatostatin	Rabbit	1/100	DakoCytomation
Transferrin	Rabbit	1/100	DakoCytomation
UDP-glucuronosyltransferase (UGT)	Sheep	1/1000	Cypex Ltd., Dundee, UK
Vimentin	Mouse	1/100	Sigma, UK
Vp16	Mouse	1/100	Sigma, UK
Vp16	Rabbit	1/250	Sigma, UK
Secondary antibody	**Species**	**Dilution factor**	**Supplier**
Anti-mouse aminomethylcoumarin acetate (AMCA) conjugated IgG	Horse	1/200	Vector Laboratories

Anti-mouse fluorescein isothiocyanate (FITC) conjugated IgG	Horse	1/200	Vector Laboratories
Anti-guinea pig FITC-conjugated IgG	Goat	1/200	Vector Laboratories
Anti-rabbit FITC-conjugated IgG	Goat	1/200	Vector Laboratories
Anti-sheep FITC-conjugated IgG	Rabbit	1/200	Vector Laboratories
Anti-sheep texas-red conjugated IgG	Rabbit	1/200	Vector Laboratories
Anti-mouse texas red-conjugated IgG	Horse	1/200	Vector Laboratories
Anti-rabbit tetramethylrhodamine isothiocyanate (TRITC) conjugated IgG	Swine	1/200	DakoCytomation
All the antibodies were diluted in 2% blocking buffer (Boehringer Mannheim, UK)			

Table 2.A.3 Antisera for Western blotting

Primary antibodies	Species	Dilution factor	Supplier
Albumin	Rabbit	1/2000	Sigma, UK
Cyp2E1	Rabbit	1/10000	a gift from Dr. Magnus Ingelman-Sundberg,Karolinska Institute, Stockholm,

			Sweden
Haptoglobin	Rabbit	1/1000	Sigma, UK
HNF1α	Goat	1/1000	Santa Cruz Biotechnology
HNF4α	Goat	1/1500	Santa Cruz Biotechnology
Transferrin	Rabbit	1/1000	DakoCytomation
α-tubulin	Mouse	1/10000	Sigma, UK
UDP-glucuronosyltransferase (UGT)	Sheep	1/30000	Cypex Ltd., Dundee, UK
Secondary antibodies	**Species**	**Dilution factor**	**Supplier**
Peroxidase-labelled anti-rabbit IgG	Goat	1/1000	Vector Laboratories
Peroxidase-labelled anti-rabbit IgG	Goat	1/2000	Vector Laboratories
Peroxidase-labelled anti-rabbit IgG	Goat	1/1000	Vector Laboratories
HRP-conjugated anti-goat IgG	Rabbit	1/1000	Abcam
HRP-conjugated anti-goat IgG	Rabbit	1/1000	Abcam
Peroxidase-labelled anti-rabbit IgG	Goat	1/1000	Vector Laboratories
Peroxidase-labelled anti-mouse IgG	Horse	1/2000	Vector Laboratories
HRP-conjugated anti-sheep IgG	Rabbit	1/3000	Abcam

Table 2.A.4 Primers for PCR

Species	Gene	Forward primer	Reverse primer	Size of PCR product (bp)	Gene back accession No.
Human	*Albumin*	CTCAAGTGTGCCAGTCTCCA	TGGGATTTTTCCAACAGAGG	272	NM_000477
Human	*Amylase*	CATCTGTTTGAATGGCGATG	TTCCCACCAAGGTCTGAAAG	138	NM_0020978

Human	*β-actin*	AGAAAATCTGGCACCACACC	GGGGTGTTGAAGGTCTCAAA	142	NM_001101
Human	*EGFR*	GGTGCAGGAGAGGAGAACTG	GGTGGCACCAAAGCTGTATT	270	AY698023
Human	*GLP1R*	GTTCCCCTGCTGTTTGTTGT	CTTGGCAAGTCTGCATTTGA	228	NM_002062
Human	*Glucagon*	CATTCACAGGGCACATTCAC	CGGCCAAGTTCTTCAACAAT	275	NM_002054
Human	*Insulin*	AGCCTTTGTGAACCAACACC	GCTGGTAGAGGGAGCAGATG	245	NM_000207
Human	*Pancreatic Polypeptide*	ACCTGCGTGGCTCTGTTACT	CAGCGTGTCCTCTTTGTGTC	180	NM_002722
Human	*Pdx1*	GAGCTGGCTGTCATGTTGAA	AGCTCCTGCCTCTCATCGT	333	NM_000209
Human	*Prohormone convertase 1/3*	TTGGCTGAAAGAGAACGGGATACATCT	ACTTCTTTGGTGATTGCTTTGG CGGTG	457	NM_033508
Human	*Somatostatin*	AGCTGCTGTCTGAACCCAAC	CCATAGCCGGGTTTGAGTTA	126	NM_001048
Human	*Transferrin*	AGAAGGGAGATGTGGCCTTT	CGACCGGAACAAACAAAAGT	296	NM_001063
Mouse	*Neurogenin 3*	CGAAGCAGAAGTGGGTGACT	GCAGGGTCTCGACCTTTGTA	236	NM_009719
Mouse	*Nkx2.2*	TCTACGACAGCAGCGACAAC	GCTTTGGAGAAGAGCACTCG	220	NM_010919
Mouse	*Nkx6.1*	ACCTTTGGGCTCACATAACC	GCGCTGGGGCTAAAGTAGAG	360	NM_144955
Rat	*β-actin*	TCCGTAAAGACCTCTATGCC	AAAGCCATGCCAAATGTCTC	477	V01217
Rat	*CYP3A1*	GGAAATTCGATGTGGAGTGC	AGGTTTGCCTTTCTCTTGCC	329	D29967
Rat	*Carbamyl phosphate synthetase I*	CGTCCAAGATTCCTTGGTGT	ATGGAAGAGAGGCTGGGATT	158	NM_017072
Rat	*Chromogranin A*	ACTAAGGTGATGAAGTGTGT	TCTCTACAGTGTCCTTGGAG	353	NM_021655
Rat	*Cyp reductase*	GGCAAGGAGCTGTACCTGAG	ATGATGACAGGTGTGGTGGA	356	M10068
Rat	*CYP2B12*	CGCATGGAGAAGGAGAAGTC	CCTCAGTGTTCTTGGGAAGC	352	X63545
Rat	*CYP7A1*	CCTCCTGGCCTTCCTAAATC	GTCAAAGGTGGAGAGCGTGT	351	NM_012942

Rat	*Glucagon-like peptide 1 receptor*	TCTCTTCTGCAACCGAACCT	CTGGTGCAGTGCAAGTGTCT	351	S75952
Rat	*IAPP*	GGCTGTAGTTCCTGAAGCTT	AAGGTTGTTGCTGGAGCGAA	260	NM_012586
Rat	*Insulin 1/2*	TGCCCAGGCTTTTGTCAAACAGCACCTT	CTCCAGTGCCAAGGTCTGAA	187	NM_019129
Rat	*Kir6.2*	ACCACGCTGGTGGACCTCAAG	GCACCACCTGCATATGAATGG	481	RNU44897
Rat	*Pdx1*	TCGCTGGGAACGCTGGAAC	CTTTGGTGGATTTCATCCACG	223	NM_022852
Rat	*Sulfonylurea receptor 1B*	AAGATCATGCACTTGTCTACT	AGACAGCAGGAACAGCGGTGT	591	AF039595
Rat	*Tryptophan 2,3-dioxygenase*	GAGCAGGAGCAGACGCTATT	CACCTTGTACCTGTCGCTCA	498	NM_022403
Rat	*Tyrosine aminotransferase*	GTCCATCGGCTACCTATCCA	CAGGACAGGATGGGAACATT	492	NM_012668
Rat	*UDP-glucuronosyltransferase 1a (UGT1a)*	ACACCGGAACTAGACCATCG	TGGAACCCCATTGCATATTC	152	NM_012683
Mouse	*Neurogenin 3*	CGAAGCAGAAGTGGGTGACT	GCAGGGTCTCGACCTTTGTA	236	NM_009719
Mouse	*Nkx2.2*	TCTACGACAGCAGCGACAAC	GCTTTGGAGAAGAGCACTCG	220	NM_010919
Mouse	*Nkx6.1*	ACCTTTGGGCTCACATAACC	GCGCTGGGGCTAAAGTAGAG	360	NM_144955
Xenopus	*Xlhbox8*	TGCCAACTTCATCCCAGCCC	GGCAGATGAAGAGGGCTC	198	X16849
All the primers were purchased from Gibco^{TM}/Invitrogen Life Technologies					

Table 2.A.5 Adenoviral reagents

Original adenovirus reagents are gifts from Dr. Harry Heimberg in Diabetes Research Center, Vrije Universiteit Brussel, Belgium.

Reagent	Stock No.	Titre (PFU/ml)
Ad CMV-HA-moNgn3-eGFP	121	$3*10^{10}$
Ad CMV-HA-Nkx6.1-eGFP	83	$4.6*10^{10}$
Ad CMV-Nkx2.2	120	$7*10^{10}$
Ad-Null	124	$1*10^{10}$
Ad TTR-Xlhbox8Vp16		$1*10^{10}$
Ad CMV-eGFP		$1*10^{10}$
Ad CMV-LacZ		$7*10^{9}$
All the adenovirus reagents were kindly provided by Dr.Mark A Hornsey and Dr.Daniel Eberhard		

Table 2.A.6 Other experimental reagent or equipment

	Supplier
Live-Dead assay	Molecular Probes
LSM 510 confocal microscope	Zeiss, Hertfordshire, UK
DMRB microscope	Leica, Milton Keynes, UK

2.B Methods

2.B.1 Cell culture

2.B.1.1 Cell passaging and maintenance

All cell lines were passaged by the same method. Prior to splitting cells, the reagents were pre-warmed to 37°C. For a 75cm^2 flask at approximately 80% confluence, the culture medium was aspirated and the cells were washed with 10 ml of sterile PBS. The PBS was then removed and 5ml of trypsin-EDTA solution (Gibco^{TM}/Invitrogen Life Technologies) was added and incubated at 37°C for 3-5 minutes to detach the cells from the flask. The same volume of supplemented medium was added to neutralise the activity of trypsin and cells were gently resuspended by gently pipetting up and down. The cells were next centrifuged at 1000 rpm in a bench top MSE mistral 1000 centrifuge (ISTCP) for 4 minutes at room temperature. The supernatant was then removed and the cell pellet was resuspended with 1 ml fresh medium and 250 µl of total suspension was plated out into a new 75cm^2 flask containing 15ml fresh complete medium (indicated in section 2.B.2). The cells were incubated at 37°C, in an atmosphere of 5% (v/v) CO_2.

2.B.1.2 Cell storage

All cells were washed and detached as previously described (see section 2.B.1.1). After the centrifugation step, the cell pellet was resuspended in 1ml freezing medium, FBS mixed with 10% (v/v) DMSO (VWR International, UK), and transferred into cryovials and kept in -20°C for about 20 minutes, followed by storage in -80°C overnight and finally transferred to liquid nitrogen for long-term storage.

2.B.1.3 Cell revival

Cells were revived by removing the cryovial from the liquid nitrogen and rapidly thawing at 37°C. The cells were then transferred into a centrifuge tube with 1ml of fresh warm medium and centrifuged at 1000 rpm for 4 minutes. The cells were then seeded in a 75cm^2 flask and cultured at 37°C with an atmosphere of 5% (v/v) CO_2. The medium was replaced with fresh medium the following day and changed every two days thereafter.

2.B.2 Primary cell culture

2.B.2.1 Isolation of the embryonic pancreas

The technique for isolation of embryonic pancreas is shown diagrammatically in Figure 2.1. Embryonic day 11.5 dorsal pancreatic tissue was obtained from female CD1 albino mice bred at the University of Bath. Adult mice were killed by cervical dislocation and the decidua removed and placed into ice-cold PBS. The embryos were dissected out and placed in Minimal Essential Medium (MEM) containing Hank's salts (Sigma, UK). The embryos were opened laterally and the stomach located. The dorsal pancreas was identified at the posterior end of the stomach and the tissue was removed with fine forceps and needles. Isolated pancreatic buds were placed cut surface down into stainless steel cloning rings on subbed and fibronectin-coated coverslips. These were in 35mm culture dishes containing 2 ml of Basal Essential Medium (BME, from Sigma, UK) with Earle's salts containing 2 mM L-glutamine, 50 µg/ml gentamycin and 20% FBS. After 24 hours of culture, the cloning ring was removed and medium was replaced with fresh medium. The medium was then changed every 2 days. Subbed coverslips (22mmX22mm; Scientific Laboratory

Supplies Ltd., UK) were prepared by rinsing with 2% (v/v) APTS in acetone overnight, followed by washing twice in acetone and sterile water. The coverslips were then dried at 37° C and heat sterilized at 180° C for at least 3 hours. The coverslips were then coated with fibronectin (Fisher Scientifics, UK) by applying 40 µl of 50 µg/ml bovine fibronectin to the centre of each coverslip and allowed to dry.

2.B.2.2 Embryonic liver cutures

The liver tissue in E11.5 embryos is easy to distinguish from other organs as the liver is the main site of hematopoiesis and is engorged with blood. The embryo was opened and the embryonic liver lobes isolated and dissected into several small pieces (one liver lobe could be split into an average of 4-6 fragments) using sharp forceps and needles. The small pieces of embryonic liver were then placed on subbed coverslips and cultured in Basal Essential Medium (BME) with Earle's salts containing 2 mM L-glutamine, 50 µg/ml gentamycin and 20% FBS for the time periods indicated in the text at 37°C in an atmosphere of 5% (v/v) CO_2. To remove the mesenchymal cells from hepatic epithelium, KSFM containing 10units/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine, 5ng/ml rhEGF, 50µg/ml BPE and FBS (with various concentration) was used.

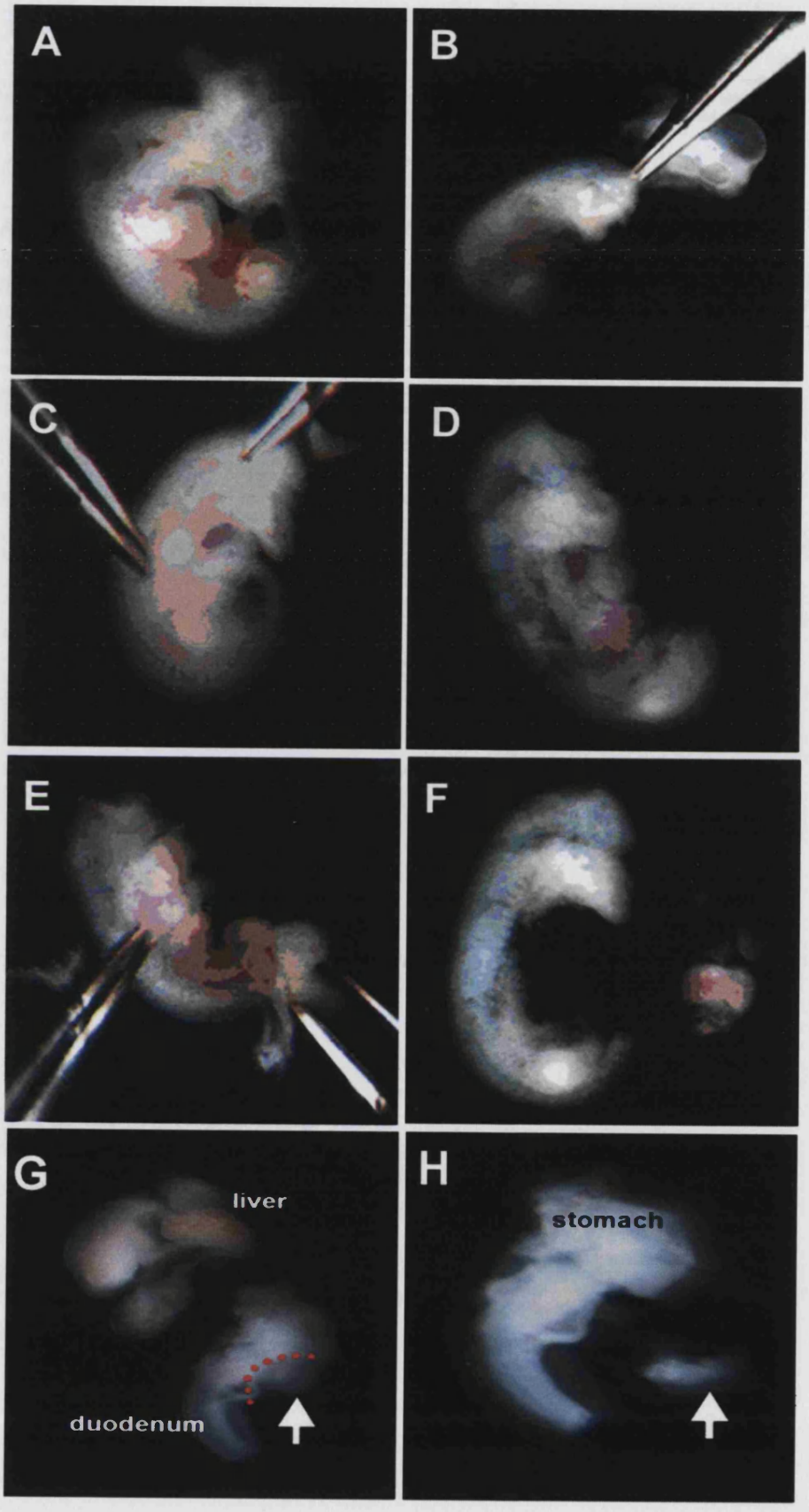

A
B
C
D
E
F
G
liver
duodenum
H
stomach

Figure 2.1 Dissection procedure for embryonic liver and pancreas from E11.5 mouse embryos. (A) The embryos were isolated from the deciduas and then placed in a dish ready for dissection; (B) The embryos were decapitated just below the lower jaw and the lower limbs were removed using fine forceps. (C) The forceps were then used to cut through the cavity after removal of the lower limbs and cut along the side of the embryo; (D) The internal organs (liver, stomach etc) were exposed; (E, F) The internal organs were removed by separating them from the back of the embryo to which they are attached; (G) Using a sharp tungsten needle, the liver was separated from the stomach. (H) The dorsal pancreatic tissue (indicated by the arrow) is located at the posterior end of the stomach and removed (indicated by the arrow).

2.B.2.3 Rat hepatocyte isolation

The technique for isolation of adult rat hepatocytes is shown diagrammatically in Figure 2.2. Male Wistar rats, weighing 270-330g, were obtained from the Animal House of the University of Bath. Hepatocytes were isolated by the two-step collagenase perfusion technique with modifications as described previously (Tosh and Collings, 1986). Briefly, the rat was anaesthetised with 4.5% isofluorane (Baxter Healthcare Ltd.) mixed with 1500 ml/minute oxygen. The rats were tested for deep anaesthesia (absence of the pedal reflex). When the rat is fully anaesthetised, the isofluorane was reduced to 3% and the animal placed dorsal side down on a tray (Fisher Scientifics). The anaesthesia was maintained via a face mask. A small amount of 70% ethanol was sprayed on the abdominal fur to reduce the risk of infection. A "U" shaped incision was then made through the skin of the lower abdomen to the lateral aspect of the rib cage and the skin was folded back over the chest. The intestines were moved aside to reveal the portal vein and the vena cava. A pair of hooked forceps were gently pushed under the portal vein and used as a guide to place a matchstick under the portal vein. Any excess adipose tissue was removed with the blunt side of the forceps and a non-absorbable suture (4-0; Ethicon™ Mersilk™) was loosely tied around the portal vein prior to inserting the cannula (18GA; Becton and Dickinson). After inserting the cannula into the portal vein the suture was tightly tied to secure its position. The inner needle of the cannula was removed and the abdominal blood vessels cut before attaching the cannula to the pump to allow perfusion medium I [0.05% (w/v) KCl (Fisher Scientific) in 10mM HEPES buffer, 5mM D-glucose (Sigma, UK), 200μM EDTA and 0.001% (v/v) phenol red (Sigma, UK) all in sterile saline pH7.4] to perfuse through the liver. The perfusion pump was set to 30-35 ml/minute. The chest cavity was then opened and the thoracic portion of the inferior vena cava was cut. The abdominal blood vessels were then clamped to ensure blood flows through

the liver. The medium was collected in the chest cavity using a syringe and discarded. After about 10 minutes perfusion at a flow rate of 30-35 ml/minute, perfusion medium I was replaced with a perfusion medium II [20mM HEPES buffer, 5mM D-glucose, 1mM $CaCl_2$ (Fisher Scientific), 0.001% (v/v) phenol red (Sigma) all in sterile saline at pH 7.4]. Approximately 150mls of collagenase-containing perfusion medium II was delivered to the liver for 10-15 minutes (with recirculation) at a flow rate of 30-35 ml/minute. For mouse hepatocyte isolation, the procedure was similar except that the perfusion flow rate is 5-10 ml/minute and the cannula is inserted into the abdominal vena cava and the portal vein is cut to allow perfusate to escape.

Following collagenase perfusion, the liver was removed and hepatocytes were dissociated using two pairs of fine forceps. The cells were filtered through a 70μm filter (Gore-Tex, USA). Dead and dying cells were removed by differential centrifugation. Cells were washed three times (50 x *g* for 2 minutes) in perfusion media II or Williams' Medium E. Using 0.4% trypan blue (w/v), the cell viability was determined to be approximately 85%. The isolated hepatocytes were initially placed in 35mm culture dishes in hepatocyte attachment medium (Williams' Medium E containing 10units/ml penicillin, 100 μg/ml streptomycin, 2mM L-glutamine, and 10% FBS). For immunohistochemistry, the cells were cultured on glass coverslips. After 6-8 hours, the attachment medium was removed and replaced with the indicated medium. The composition was as follows: Williams' Medium E (W), Williams' Medium E plus dexamethasone (WD), Williams' Medium E plus supplement (5ng/ml rhEGF and 50μg/ml BPE, from Gibco™/Invitrogen Life Technologies) (WS) and Williams' Medium E plus supplement plus 1μM dexamethasone (WDS), KSFM (K), KSFM plus 1μM dexamethasone (KD), KSFM plus 5ng/ml rhEGF (KE), KSFM plus 50μg/ml BPE (KB), KSFM plus supplement (KS) and KSFM plus supplement plus 1 μM dexamethasone (KDS).


A Equipments
Tubing
Anaesthetising chamber
Peristaltic pump
Dissection tray
Dissection instruments
Cannula
B
C
Dissocation of hepatocytes

Figure 2.2 Procedure for hepatocyte isolation (using Wistar rat as an example here). (A) Equipment for hepatocyte isolation. See details described in chapter 2.B.2.3.; (B) Flow chart for isolating hepatocytes. Checking the mouse is fully anaesthetised by using the pedal reflex and U-shapely opened the abdomen of the mice (B.1-B.5); removing the excess fat tissues to expose the portal vein and loosely tying a suture around the vena cava prior to inserting the cannula (B.6, B.7); inserting the cannula into the vena cava, removing the inner needle of the cannula and securing with the suture (B.8-B.10); introducing perfusion medium I to perfuse through liver (B.11); meanwhile, opening the chest cavity and clamping vena cava (B.12-B.14); after 10 minutes perfusion, changing from perfusion medium I to perfusion medium II for another 10 minutes and removing the liver when it was adequately digested (B.15, B.16); (C) Procedure for the preparation of dissociated liver cells. Dissociating liver lobes using two pairs of forceps and filtrating the homogenised liver cells through a gauze mesh filter.

2.B.3 Plasmid construction

The reagents for molecular cloning, plasmid maps and construction strategies are all shown in appendix 1. In general, the indicated restriction enzymes were incubated with 1-2 μg plasmid DNA at 37°C (unless otherwise stated) for 1.5-2 hours. The digested products were then separated by electrophoresis on a 1-1.5% agarose gel and the DNA extracted from the gel using a DNA extraction kit (section 2.A.3) according to the manufacturer's instructions. To produce blunt ended plasmid DNAs, the DNA was incubated with Klenow fragment (1 unit per mg DNA) at 30°C for 20 minutes. The reaction was stopped by the incubation of samples at 70°C for 15 minutes. The vector was treated with CIP to catalyse the removal of 5′ phosphate groups from DNA. Since CIP-treated fragments lack the 5′-phosphoryl termini required by ligases, they cannot self-ligate. Afterwards, 1:3-1:5 (vector/insert ratio) of DNA was mixed with 1X buffer and 0.5 units T4 DNA ligase at 20°C for overnight for ligation reaction. The ligation products were then incubated with several different restriction enzymes to ascertain whether the ligated fragment was in the correct orientation.

2.B.4 Gene delivery

2.B.4.1 Transfection

Transfection of plasmids into cells was tested with various transfection reagents including GeneJuice (Novagene, USA), Lipofactamine 2000 (Gibco^{TM}/Invitrogen Life Technologies), Exgene 500 (Fermentas) and Fugene 6 (Roche, UK) according to the manufacturer's instructions. Among them, GeneJuice transfection reagent exhibited the optimal condition for transfection of plasmid DNA into cells (Figure 2.3). Briefly, for

each reaction, 3µl GeneJuice reagent was mixed with 100µl serum free medium and allowed to stand at room temperature for 5 minutes. The same amount of plasmid DNAs (usually 1-2 µg) were then added into the GeneJuice-medium mixture and incubated for 20-30 minutes. Lastly, for HepG2 and Panc-1 cells, the GeneJuice-DNA mixture was evenly distributed over the cells. For transfection of liver buds, GeneJuice was added directly onto the tissue whilst in a cloning rings.

2.B.4.2 Adenoviral infection

HepG2 cells were seeded onto 35mm dishes at a density of $1X10^6$. Rat hepatoctyes were plated onto culture dishes at a density of about $5X10^5$ (for 35mm dishes) or $1X10^6$ (for 60mm dishes). To test the adenoviral infection efficiency in HepG2 cells, the stock Ad CMV-GFP virus (an adenovirus containing the GFP gene under the control of the CMV promoter) was diluted at 1:100, 2:100 and 3:100 in sterile PBS. Cells were infected with the infection concentration of 100, 200 and 300 MOI (multiplicity of infection, the number of viral particles taken up by each individual cell). The infected cells were incubated at 37° C for 1 hour and the medium was then changed to fresh medium. The cells were maintained for 2 days before fixation for immunostaining.

Infection of embryonic liver cells was carried out by the same procedure as for infection of HepG2 cells. However, the total virus load (with various dilutions) was deposited in the cloning ring. For viral infection of rat hepatoctyes, the infection was carried out after the cells had been in culture for 72 hours. For rat hepatocytes, the titres tested were 200, 20, and 2 MOI. In the combination experiments, the total viral titre was about 20 MOI, that is to say, infecting $1X10^7$ or $2X10^7$ total viral particles onto 35mm and 60mm dishes, respectively.

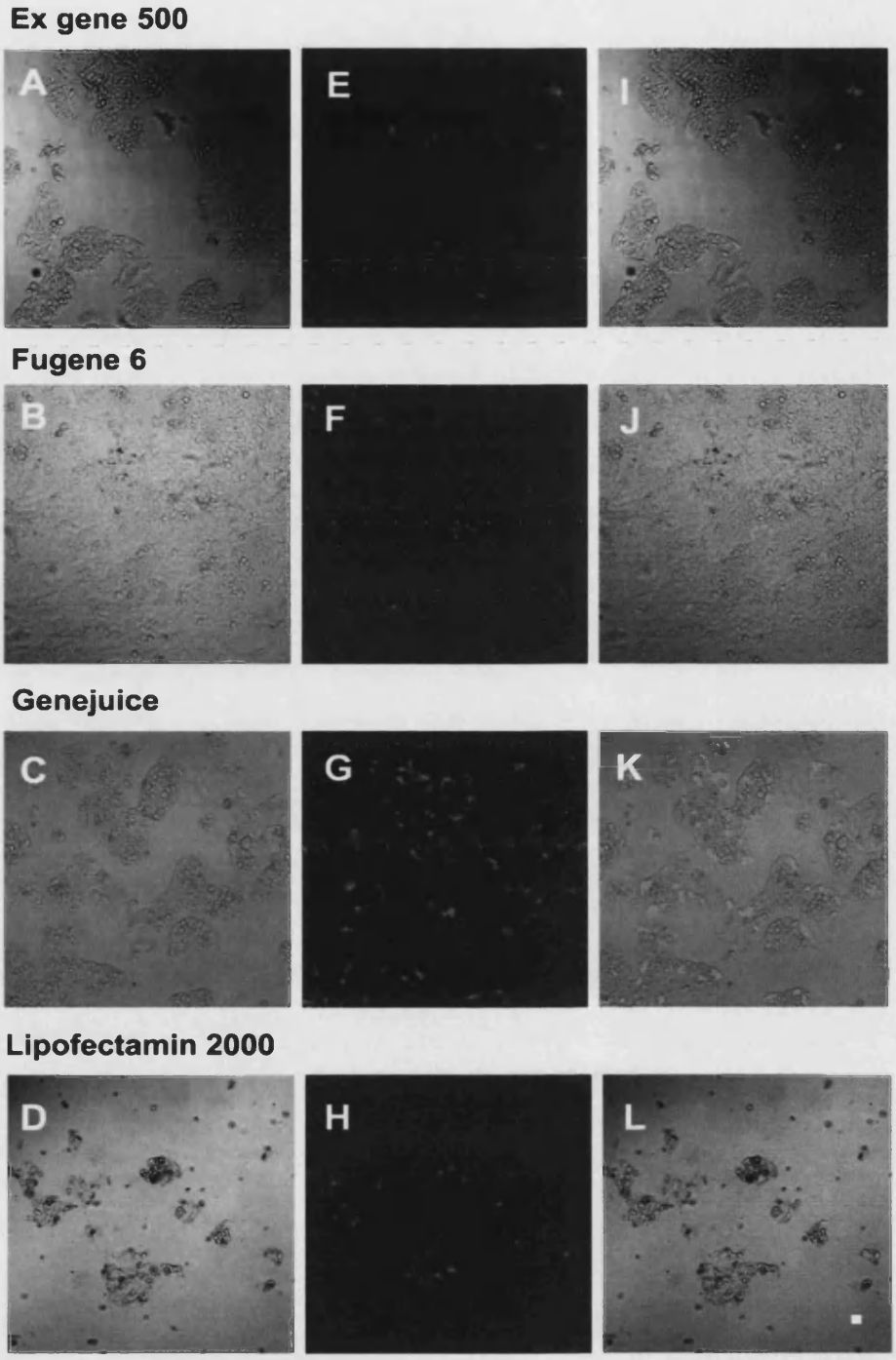


Figure 2.3 Comparison of transfection efficiency of HepG2 cells with different transfection reagents. HepG2 cells were transfected with 1μg CMV-nGFP plasmid DNA according to the manufacturer's instructions. After 24 hrs, transmitted light images (A-D) and fluorescent images (GFP expression) (E-H) were collected. Merged images are also shown (I-L). Scale bar = 20 μm

2.B.5 Qualitative and quantitative RT-PCR

2.B.5.1 RNA extraction

Total RNA was extracted from cells using the TRI reagent (Sigma, UK) following the manufacturer's instructions. Digestion with RQ1 RNase-free DNase (Promega) was performed to eliminate any contaminating genomic DNA. The concentration of extracted RNA was measured on a Beckman Spectrophotometer. The ratio of 260nm to 280nm of each sample ranged from 1.8 to 2.0.

2.B.5.2 Reverse trancription

Messenger RNA was isolated from total RNA by using SuperScript™ First-Strand Synthesis System for RT-PCR. Briefly, the messenger RNA (mRNA) was first isolated by incubation of Oligo$(dT)_{12\text{-}18}$ oligonucleotides from 5-10 µg extracted total RNA. Complementary DNA (cDNA) was prepared by SuperScript™II reverse transcriptase treatment. cDNA samples were incubated with 2 units RNase H at 37° C for 20 minutes to remove the RNA-cDNA hybridised residues. All the reagents were from Gibco™/Invitrogen Life Technologies.

2.B.5.3 PCR reaction

Polymerase chain reactions containing the mixture of the same concentration of cDNA, 1X PCR buffer, 1.5mM $MgCl_2$, 0.2mM dNTP, 50 ng sense/antisense primers and 1.25 U Platinum Taq polymerase were processed in a DNA thermal cycler using the following conditions: denatured at 94° C for 1 minute, amplification at 58° C for 1 minute and elongation at 72° C for 1 minute for 30-40 cycles. The samples were analysed in 1.2~1.5% agarose electrophoresis with 1kb ladder marker.

To obtain more quantitative results for the different samples, we also performed real-time PCR using the Lightcycler system (Roche). The same amount of cDNA from calibrator and experimental cell extracts was mixed with 1XSYBR@ Green Taq ReadyMix™ (Sigma, UK), 50 ng sense and antisense primers and transferred into Lightcycler capillaries. The following conditions were used for the amplification of PCR products: denaturation at 95°C for 30 seconds; amplification at 58°C for 5 seconds followed by 72°C for 20 seconds for 50 cycles; cooling at 40°C for 2 minutes. The fluorescence signal was detected at the same time point of each cycle. Data were presented as the normalised ratio, which is (Conc.$_{[\text{target gene (experimental)}]}$ / Conc.$_{[\text{reference gene (experimental)}]}$):(Conc.$_{[\text{target gene (control)}]}$ / Conc.$_{[\text{reference gene (control)}]}$) by Lightcycler Relative Quantification software.

2.B.6 Immunofluorescence staining

For immunofluorescence staining, the cells were rinsed with PBS to remove any excess medium and then fixed with 4% (w/v) paraformaldehyde (PFA, VWR International) in PBS for 20-30 minutes, the cells were then permeabilised with 0.1% (v/v) Triton X-100 (Sigma, UK) in PBS for 30 min. For the preparation of 100ml 4% PFA, 4 gm PFA was added into 85 ml lukewarm PBS. The pH was then adjusted to 7.4 by adding 10M NaOH and concentrated HCl. The volume was then topped up with PBS. In contrast to cells, primary cultures of embryonic tissues were fixed in MEMFA for 20-30 minutes. MEMFA was prepared by mixing the following reagents: 10% v/v formaldehyde (Sigma stock concentration = ~38% so final concn is about 4% w/v), 0.1 M MOPS (pH 7.4) (Sigma), 2mM ethylene-glycotetraacetic acid (EGTA, Sigma) and 1mM $MgSO_4$ (Sigma). Tissue was permeabilised with 1% Triton X-100 in PBS for 30 minutes. For the antigens including connexin 32, E-cadherin and smooth muscle actin,

the cells or tissues were fixed with acetone-methanol (1:1; v/v) fixatives at -20°C for 5-8 minutes, permeablised with 1% Triton X-100 in PBS for 30 minute and then incubated in 1X citrate buffer (Lab Vision Corporation, CA) at 37° C for 30 minutes before blocking. Afterwards, cells or tissue were incubated in 2% blocking buffer for at least 1 hour. The coverslips were then incubated with primary antibody overnight at 4° C. On the following day, the coverslips were washed with PBS. The fluorescently-conjugated secondary antibodies were added to the coverslips for 3 hours and then washed with PBS. The slides were mounted with Gel/Mount mounting medium (Biomeda, Foster City, CA). If there was more than one antigen to be detected, the primary and secondary antibodies were added sequentially using the same protocol described above. For cell counting experiments, cells were incubated with antibodies followed by incubation with 500ng/ml 4,6-Diamidino-2-phenylindole (DAPI; Sigma, UK) for 20 minutes before mounting.

2.B.7 Insulin ELISA

2.B.7.1 Sample collection

Cells were cultured for 5 days and transfected with TTR-VP16 or TTR-Xlhbox8Vp16 and treated with medium containing either normal glucose (5.5mM) or high glucose (25mM) for 12 hours. The media was concentrated by using Centricon YM-3 Centrifugal Filter Devices (Millipore Corporation, nominal molecular weight limit 3 KiloDalton). 15-20 ml medium were applied into the sample reservoir and centrifuged at 6500*xg* until all the medium had been filtered. The concentrated samples were collected into retentate vials by inverting the unit and centrifuging at 1000*xg* for 5 minutes.

2.B.7.2 Protein concentration analysis

The concentration of total protein was assayed by the Bio-Rad protein reagent (Bio-Rad Laboratories). Briefly, a 1:1000 dilution of the sample and standard (0-25 μg/μl bovine serum albumin; Pierce Biotechnologies Inc.) proteins were mixed with 1/5 volume of assay reagent and incubated at room temperature for 5 minutes. The optical density was then measured at 595nm. The values were blank corrected and a standard curve was plotted of absorbance against protein concentration. The protein sample concentration was determined from the standard curve.

The insulin concentration in the medium was analysed using a rat Insulin ELISA kit (Mercodia AB) following the manufacturer's instruction. The samples and standards (recombinant human insulin; Sigma, UK), which were different concentrations of recombinant human insulin solution diluted in distilled H_2O, were performed in triplicate for each set of assays.

2.B.8 Histochemistry

2.B.8.1 Oil red O staining

To detect lipid storage in cultured rat hepatocytes, oil red O staining was performed. The rat hepatocytes were cultured for various time periods and fixed with MEMFA for 20-30 min. The fixed cells were pre-treated with 60% (v/v) 2-propanol for 2 minutes and then incubated in oil red O staining solution (prepared by dissolving 1% (w/v) oil red O in 60% 2-propanol solution) for 10 minutes. The staining solution was incubated at 100°C, cooled down to room temperature and filtered through 0.22μm syringe filter prior to use. The cells were then post-treated with 60% (v/v) 2-propanol for another 30 seconds and washed with running tap water for 10-20 seconds.

Counter staining of cell nuclei was carried out by incubating the cells in Ehrich's haematoxylin solution (Raymond A LAMB limited) for 1 minute. The cells were mounted in DePeX mounting medium. The images were visualized using the 40X objective lens of a Leica DMRB microscope and photographed with a digital spot camera.

2.B.8.2 Periodic acid - Schiff (PAS) staining

The PAS assay was performed to detect glycogen storage (Lazaro et al., 2003). The reaction is based upon the fact that periodic acid oxidise 1,2-glycol groups to produce aldehydes which form a coloured reaction product with Schiff's reagent (Derenzini et al., 1986). Briefly, the cells were seeded on the coverslips and culture for 1 / 2 / 3 and 4 weeks in KDS medium and then incubated with KDS plus 25mM glucose for the following 24 hours. The cells were fixed with 4% PFA and then permeabilised with 1% Triton X-100 at room temperature for 20-25 minutes. The cells were washed with tap water for 1-2 minutes and transferred to 1% periodic acid solution for 30 minutes. Next, the cells were washed with running tap water for 3 minutes and then incubated in the Schiff's reagent at room temperature for 30 minutes to develop the red colour showing the expression of glycogen. After washing in running tap water for 10 minutes, the slides were mounted with Gel/Mount aqueous mounting medium.

2.B.9 Western blotting

2.B.9.1 Preparation of the cell lysate

Cells were harvested by washing 3 times in cold PBS and detached by applying

trypsin-EDTA for approximately 3 minutes. The cells were centrifuged at 1000 rpm for 4 minutes and the cell pellet was then suspended with 100µl lysis buffer [150mM NaCl, 1mM EDTA, 2mM dithiothreitol (DTT; Sigma, UK), 20mM HEPES (pH 7.6) and 1%(v/v) Triton X-100] containing 1/100 dilution of protease inhibitor cocktail (Sigma, UK). The lysate was kept on ice for 10 minutes then centrifuged at 13000 rpm in a bench top MSE Micro Centaur centrifuge (ISTCP) at 4°C. The supernatant was transferred into a new tube and stored in -80°C until use. To extract the protein from the whole tissues, the tissue was homogenised using a plastic homogeniser (Fisher Scientifics) in lysis buffer containing 1/100 dilution of protein inhibitor cocktails and following the same procedure described above.

2.B.9.2 Protein quantification assay

The proteins were measured using Bio-Rad protein assay (see section 2.B.7.2).

2.B.9.3 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

10-15µg total protein was mixed with the same volume of 2X sample loading buffer [125mM Tris-HCl (pH 6.8), 4%(v/v) SDS, 20% (v/v) glycerol (Sigma, UK), 0.2mM DTT and 0.02% (v/v) bromophenol blue (Sigma, UK)] and denatured by heating to 100°C for 5 minutes. The proteins with molecular weight standards (Bio-Rad Laboratories) were then separated in 10% or 15% Criterion™ pre-cast Tris-HCl polyacrylamide gel (Bio-Rad Laboratories) at constant voltage of 80-100. Prior to the Western blot transfer, the gel, nitrocellulose (Pall Corporation, Pensacola FL), filter paper (Amersham Bioscience) and support pads were soaked for 15 minutes in transfer buffer [pH8.3, prepared by mixing 25mM Tris, 192mM Glycine (Sigma, UK) and 20% methanol (Fisher Scientifics)]. The Western blot cassette was

assembled according to manufacturer's protocol. Air bubbles were removed by rolling a tube on top of the sandwich. The whole cassette was placed in the blotting chamber and enough transfer buffer was added to submerge it. The blotter was then connected to a power supply and the transfer was run at 200mA for 2 hours.

2.B.9.4 Immunoprobing and signal detection

The nitrocellulose paper was stained with diluted Ponceau S [prepared by mixing 1% (w/v) Ponceau S (Sigma, UK) and 5% (v/v) CH_3COOH (Fisher Scientifics) and diluted with distilled water in a ratio of 5:9] for about 1 minute and destained with distilled H_2O to visualise the protein. The Ponceau S was totally removed by washing in 0.1% (v/v) PBS-Tween20 (PBST; Tween-20 is from Sigma, UK). To reduce non-specific background, the membrane was blocked with 5% (v/v) non-fat milk in 0.1% (v/v) PBST at 4°C overnight. For the following stages, the blot was incubated at room temperature with gentle constant agitation on a rocker (Jencons Scientific Ltd., UK). All antibodies were diluted in 3% (v/v) non-fat milk in 0.1% (v/v) PBST. The membrane was probed with primary antibodies, at the dilutions shown in the text, for 2 hours. The blots were then washed with PBST 3 times for 15 minutes and incubated with diluted secondary antibodies for 1 hour. After the last 15-minute wash with PBST, the signals on the membrane were detected with the ECL™ Western blotting analysis system (Amersham Bioscience) by incubating the membrane with 1:1 mixed ECL reagents and developed on Hyperfilm™ (Amersham Bioscience) by X-ray processing.

2.B.10 Urea cycle assays

Two colourimetric assays, designed to detect secretion of urea and activity of arginase, were carried out on the cultured rat hepatocytes.

2.B.10.1 Urea assay

The urea assay was performed based on a previously published protocol (Meng et al., 2004) with minor modifications. Briefly, 100µl of urea standards (0-50µg/ml; Gibco^{TM}/Invitrogen Life Technologies) and 24-hour culture medium were prepared and incubated with 300µl urease buffered solution at room temperature for 20 minutes. 600µl of phenol nitroprusside, 600µl of alkaline hypochlorite and 3ml distilled H_2O were then added, gently mixed and incubated at room temperature for 30 minutes. The absorbance of standards and samples were then measured at an OD of 630nm.

2.B.10.2 Arginase assay

The arginase assay was based on a method by Corraliza et al (Corraliza et al., 1994) with minor changes. The urea production was detected at OD 540nm. Urea production from the hydrolysis of arginine by arginase was taken as proportional representation of endogenous arginase activity. In short, the cultured rat hepatocytes were lysed in 0.1% (v/v) Triton X-100 containing a 1/100 dilution of protease inhibitor cocktail and shaken for 30 minutes. The lysate was then mixed with same volume of 25mM Tris-HCl (pH 7) containing 5mM $MnCl_2$ and the enzyme was activated by incubation at 56°C for 10 minutes. 25µl of activated lysate was then incubated with 25µl of 0.5M L-arginine at 37°C for 1 hour. The samples and the urea standards (0-500µg/ml) were then incubated with 400µl of an acid solution comprised of H_2SO_4 (98%; VWR International), H_3PO_4 (88%; Fisons scientific apparatus) and H_2O at a

ratio of 1:3:7 and 25µl 9% (w/v; dissolved in 100% ethanol) ISNP at 100°C for 45 minutes.

2.B.11 Live-dead assay

The live-dead assay was performed using LIVE/DEAD® Viability/Cytotoxicity Assay. It provides a two-colour fluorescence cell viability assay that is based on the simultaneous determination of live and dead cells with two probes that measure recognized parameters of cell viability – intracellular esterase activity and plasma membrane integrity. The live cells are determined by the generation of green fluorescence by the breakage of a nonfluorescent cell-permeant calcein AM dye into fluorescent calcein. This reaction is only active in cells with intracellular esterase, namely live cells. The ethidium homodimer dye can enter into cells while the cell membrane is damaged, i.e. apoptotic cells or necrotic cells (Lucas et al., 1998; Pulliam et al., 1998; Reader et al., 2003), and undergo enhancement of fluorescence upon binding to nucleic acids (Rye et al., 1991), thereby producing a bright red fluorescence in the dead cells.

Briefly, the cells were treated with 320nM calcein AM and 1.6 µM ethidium homodimer reagents and incubated at 37°C for 20-40 minutes. The cells were then washed with sterile PBS for 3 times and checked under the fluorescence microscope.

2.B.12 Image processing

Fluorescent specimens were examined and images collected under a Zeiss LSM 510 confocal microscope. Images were processed with Adobe Photoshop 7.0. For cell counting experiments, numbers of random fields were selected using the 40X objective lens of a Leica DMRB microscope. The cell numbers were visualised by

4,6-diamidino-2-phenylindole (DAPI staining). DAPI was dissolved in PBS at 500 μg/ml, and used at a dilution of 1:1000. Coverslips were incubated with DAPI (500 ng/ml) for 30 min at room temperature before being mounted on to slides in Gel/Mount mounting medium.

2.B.13 Statistics

The data were expressed as mean ± standard deviation. Comparison of individual treatments was conducted using Student's t-test.

Chapter 3.

In vitro conversion of human hepatoma HepG2 cells into pancreatic-like cells

Overview

It has been previously demonstrated the conversion of liver cells to pancreatic cells in two model systems: *Xenopus laevis* and human HepG2 cells. In both models conversion of liver to pancreas was brought about by overexpression of a modified form of Pdx1 (Horb et al., 2003). While these results were informative, we wished to investigate in more detail the transdifferentiation event. The aim of the research outlined in this chapter is two-fold. First we are determining the phenotype of the liver cells and pancreatic cells. Second, we wanted to determine the cell lineage to find whether transdifferentiation was really occurring. It has been applied to the conversion of HepG2 cells to pancreatic cells the criteria of Eguchi and Kodama for transdifferentiation (see discussion in section 1.B). The first criterion is to demonstrate the phenotype of the parent cell and the daughter cell either by protein expression (e.g immunohistochemistry) or RNA expression (by RT-PCR). The second criterion is to show the ancestor-descendant relationship between the two cell types. We also asked whether (i) the presence of the Vp16 domain affects the transdifferentiation and (ii) the transdifferentiated pancreatic cells induced from HepG2 cells can divide?

3.A Differentiated properties of HepG2 cells

The HepG2 cell line was derived from a liver biopsy of a male Caucasian (aged 15 years)(Aden et al., 1979). It is a well-differentiated hepatocellular carcinoma and has been shown to secrete a range of liver serum proteins (Aden et al., 1979; Dashti and Wolfbauer, 1987). To examine the differentiated properties we determine the expression of liver-specific proteins by immunostaining. The results showed that HepG2 expressed liver-specific proteins (albumin, α1-antitrypsin, ceruloplasmin, haptoglobin and UDP-glucuronoyltransferase) and liver-enriched transcription factors

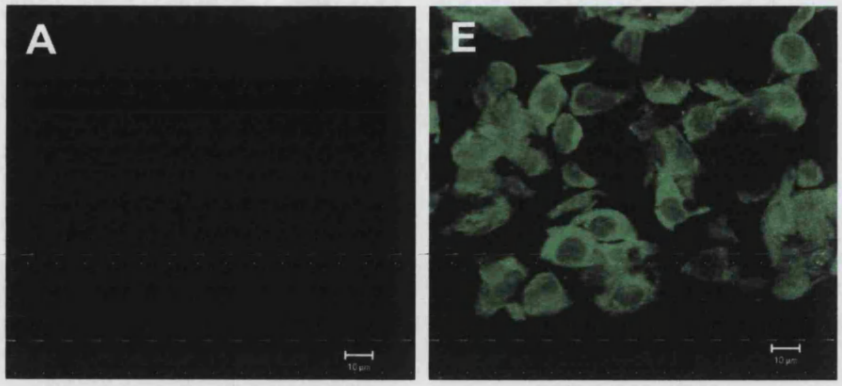
(C/EBPβ, HNF4α and RXRα) similar to normal hepatocytes (Figure 3.1). The results suggested that HepG2 cells are differentiated cells and therefore may be used to investigate transdifferentiation

3.B Molecular examinations for transdifferentiation

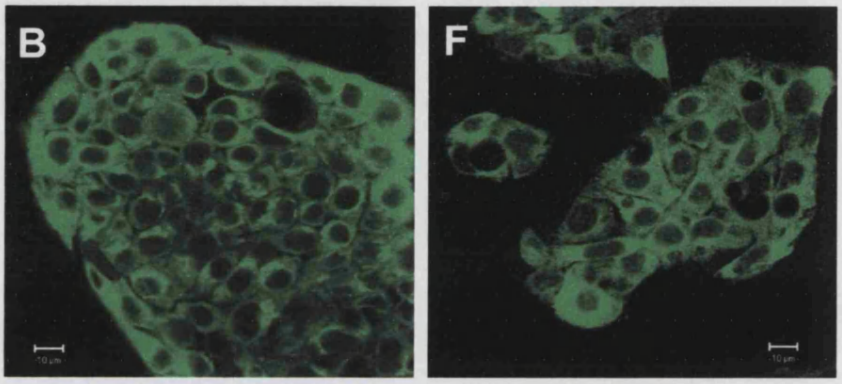
3.B.1 TTR-Xlhbox8Vp16; EI-GFP transfection in HepG2 cells

We wished to extend our previous observations on the conversion of liver to pancreas in HepG2 cells to examine the transdifferentiation event in more detail in molecular and cellular aspects (Horb et al., 2003). We transfected the pCS2-TTR-Xlhbox8Vp16; EI-GFP plasmid into HepG2 cells. In principle, it would be expected that the TTR promoter is active in HepG2 cells because transthyretin is expressed (Figure 3.2) (Costa et al., 1986). This would drive expression of Xlhbox8Vp16.

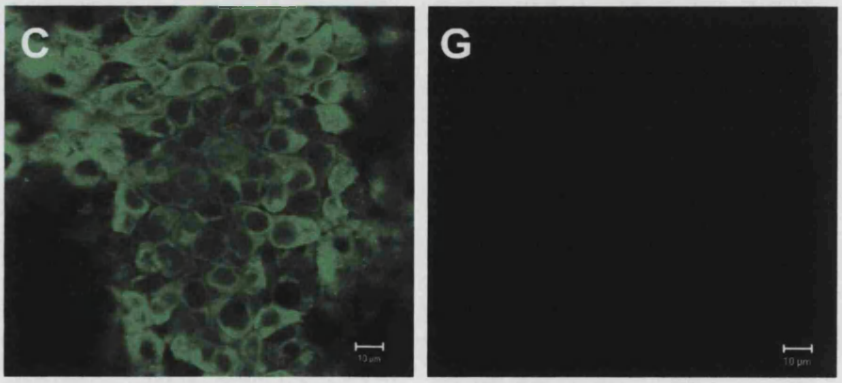
However, once a cell starts to express Xlhbox8, it will acquire a pancreatic identity. When the HepG2 cells convert to a pancreatic cell, the TTR promoter will shut down and the pancreas-specific elastase promoter will be activated. Since the region of the elastase promoter used in the present studies is active in both exocrine and endocrine cells activation of the promoter will only indicate conversion to a pancreatic phenotype and not specifically exocrine differentiation (Kruse et al., 1993). We determined the expression of Vp16 (which is controlled by TTR promoter) and GFP (which is controlled by the elastase promoter) by immunofluorescence staining.



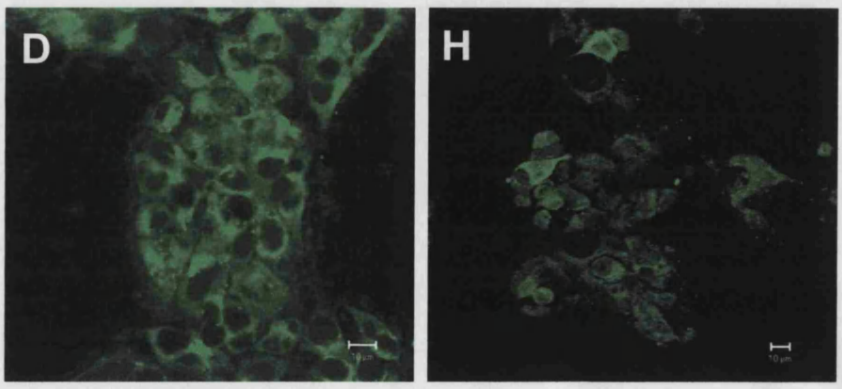
Acyl sulphotransferase Ceruplasmin



α1-fetoprotein Haptoglobin



Albumin Multiple drug resistant protein



α1-antitrypsin UDP-glucuronosyltransferase

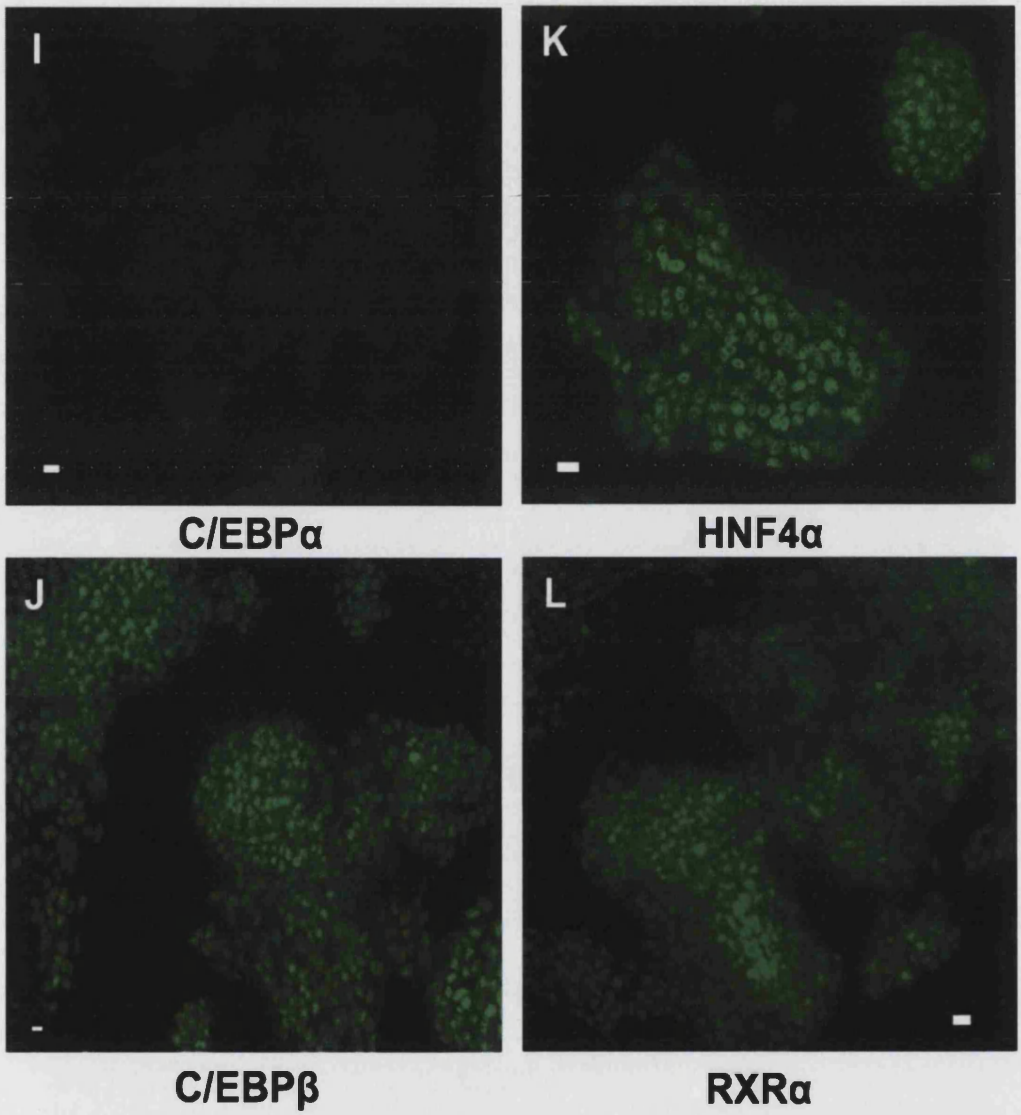


Figure 3.1 Characterisation of the liver phenotype in HepG2 cells. Indirect immunofluorescence staining was performed on HepG2 cells. The hepatic proteins (A) aryl sulphotransferase; (B) α1-fetoprotein; (C) albumin; (D) α1-antitrypsin; (E) ceruloplasmin; (F) haptoglobin; (G) multiple durg resistant protein; (H) UDP-glucuronosyltransferase and liver enriched transcription factors (I) C/EBPα; (J) C/EBPβ; (K) HNF4α and (L) RXRα were detected in HepG2 cells. Scale bar: (A-H) 10 μm; (I-L) 20 μm.

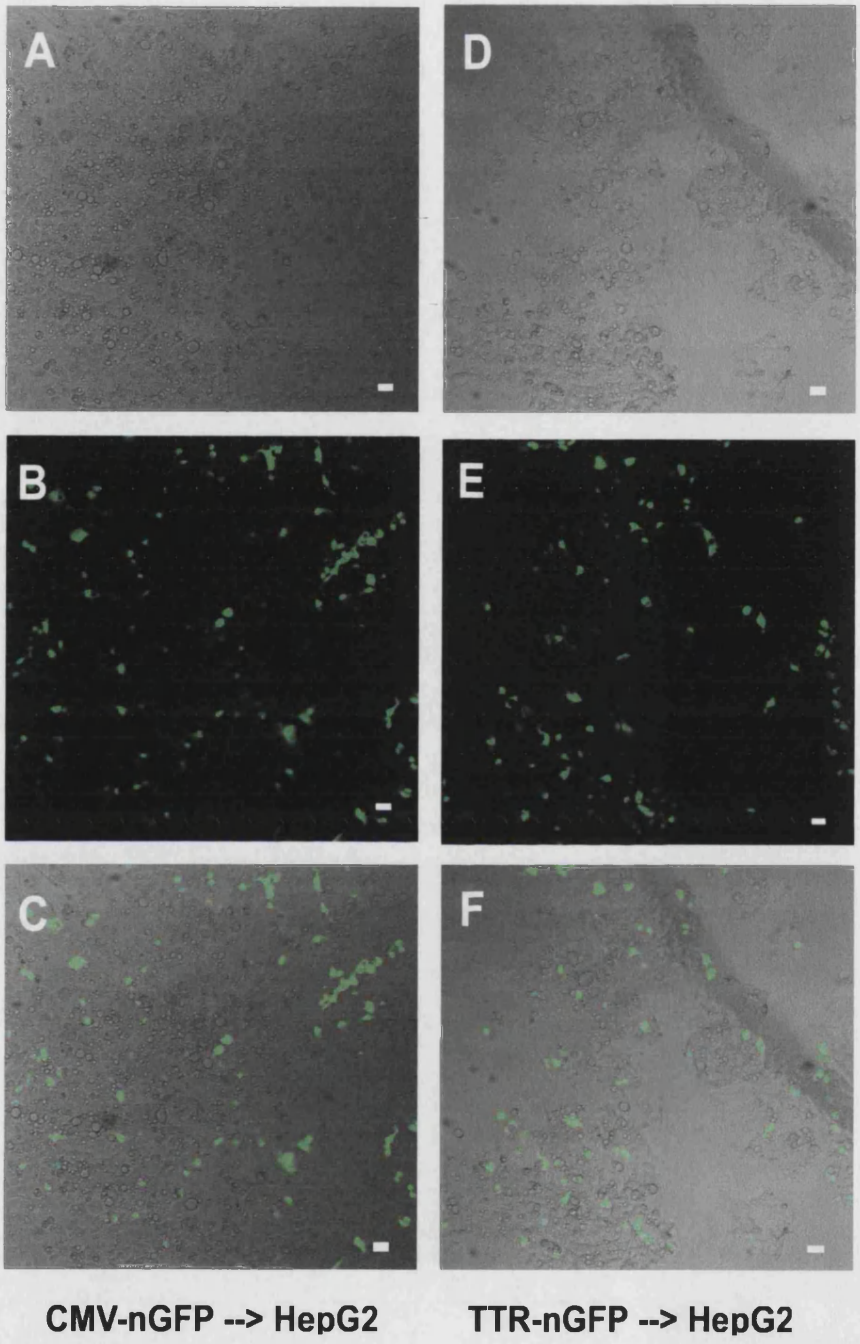


Figure 3.2 Transfection of (i) pcDNA3-CMV-nGFP and (ii) pcDNA3-TTR-nGFP plasmids into HepG2 cells. The result showed similar transfection efficiency of these constructs in HepG2 cells indicating the ubiquitous expression of TTR promoter activity in HepG2 cells. (A,D) transmitted images; (B,E) GFP expression; (C,F) merged images. Scale bar = 20 μm.

Results showed that GFP expression was activated from day 1 post transfection (Figure 3.3). Co-staining for Vp16 and GFP expression in the converted cells demonstrated time-dependent changes. The results show that Vp16 expression gradually decreased while GFP expression increased from the 1st to the 5th day after transfection (Figure 3.4). The cells displaying expression of only one of the two products show a crossover in frequency between two and three days after transfection although the majority of the cells express both Vp16 and GFP proteins.

This result is consistent with the proposed mechanism, but a surprisingly high percentage of pCS2-TTR-Xlhbox8Vp16; EI-GFP transfected cells co-localised Vp16/GFP on 1st day after transfection. Further investigation revealed that the elastase GFP reporter system is to some extent active in the control HepG2 cells so there is a significant background of GFP positive cells. Figure 3.5A, 3.5B shows control experiments done with pCS2-TTR-Vp16; EI-GFP and pIT2-EI-GFP. This problem was further exemplified by qualitative data on the co-staining for insulin and GFP or amylase and GFP, which showed an extremely low percentage of cells expressing both pancreatic markers and GFP (Figure 3.6). In addition, control experiments including the detection of GFP protein in (transgene null control) transfected cells or the unlabelled GFP in insulin/amylase producing cells (Figure 3.7C, 3.7D) were demonstrated. An interesting observation was found that GFP was not detected in HepG2 cells while the Elastase-GFP reporter cassette is put in a pcDNA3 backbone (Figure 3.5C). These data confirmed that the pCS2-EI-GFP reporter system is not sufficiently reliable for following the expression of the pancreatic phenotype in HepG2 system. Based on these results, it was decided to remove EI-GFP from pCS2-TTR-Xlhbox8Vp16; EI-GFP plasmid to make a new construct: pCS2-TTR-Xlhbox8Vp16 for the remaining experiments (see Appendix 1). In parallel, a negative control construct was generated: TTR-Vp16 which lacked the

functional Xlhbox8 sequence.

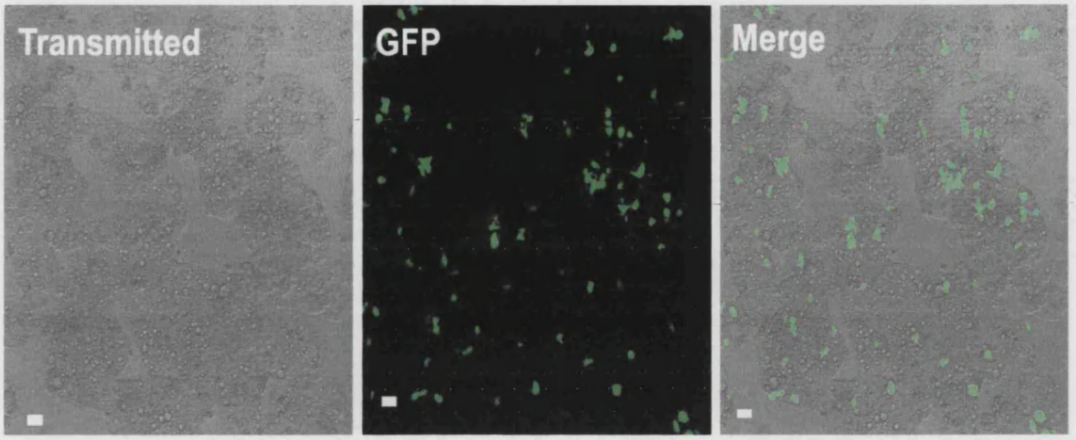


Figure 3.3 GFP expression in pCS2-TTR-Xlhbox8Vp16; El-GFP transfected HepG2 cells after 24 hours. The expression of GFP protein reflects the activation of TTR promoter. Scale bar = 20 μm.

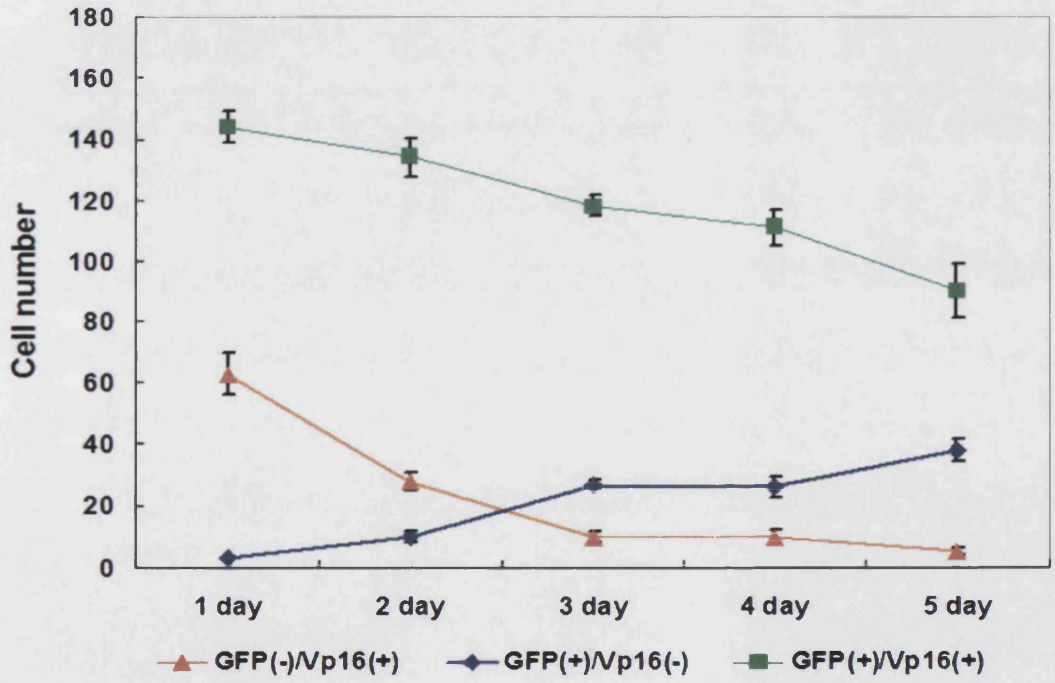


Figure 3.4 Time-dependent expression of pancreatic (elastase) and hepatic (TTR) promoters in the HepG2 cells transfected with pCS2-TTR-Xllhbox8Vp16; elastase-GFP. The gradual increase of GFP(+) / VP16(-) and decrease of GFP(-) / VP16(+) cells indicates that there is a dynamic process occurring in the conversion of hepatic cells to pancreatic cells.

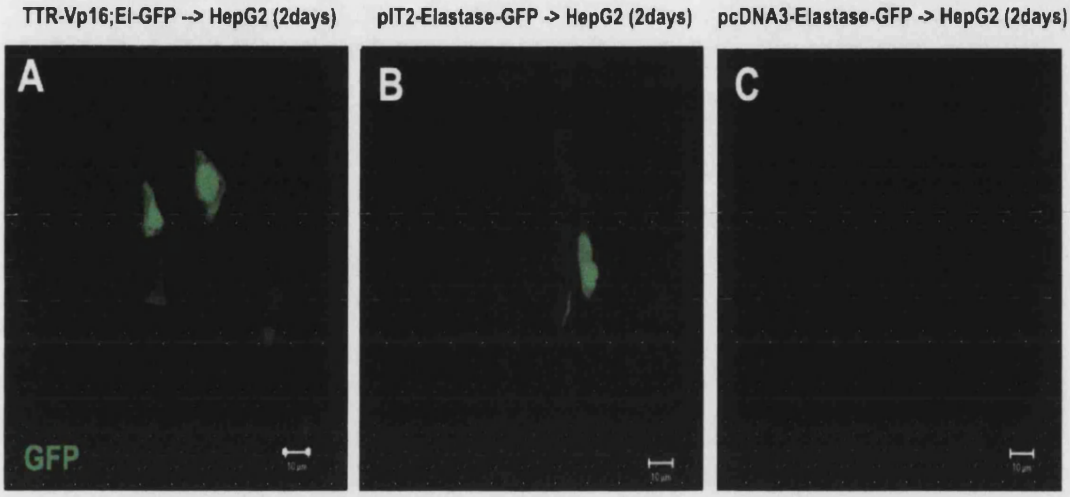


Figure 3.5 The detection of GFP expression in (A) pCS2-TTR-Vp16; EI-GFP, (B) pCS2-Elastase-GFP or (C) pcDNA3-Elastase-nGFP transfected HepG2 cells. The GFP expression was determined on the 2nd day after transfection. Scale bar = 10 μm.

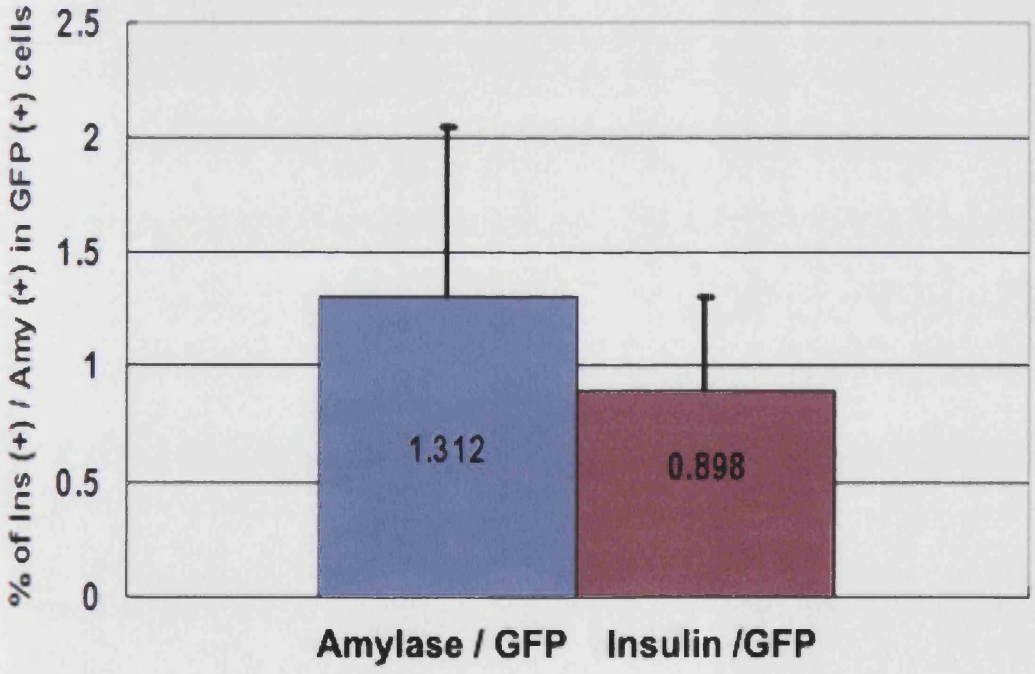


Figure 3.6 The percentage of the amylase or insulin producing cells among GFP positive cells after 5 days of pCS2-TTR-Xlhbox8Vp16; EI-GFP transfection in HepG2 cells. The results are presented as mean+S.D. (n≥6)

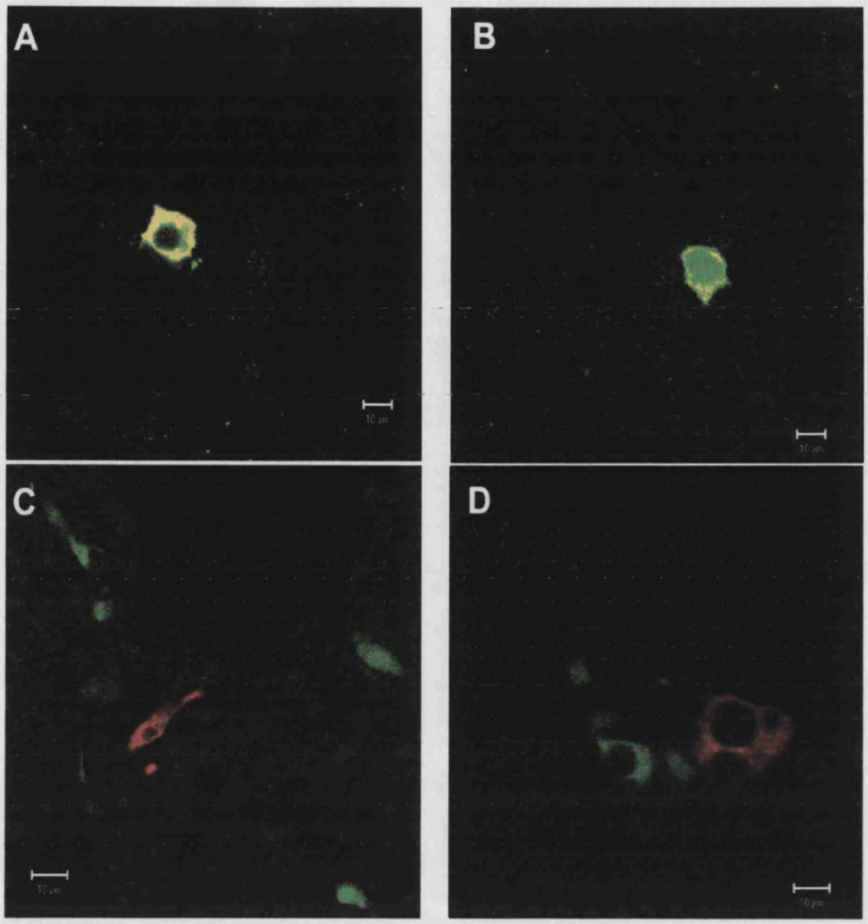


Figure 3.7 Dual staining of GFP and amylase or insulin on the HepG2 cells transfected with pCS2-TTR-Xlhbox8Vp16; EI-GFP plasmid for 5 days. The amylase or insulin positive cells were either co-stained with GFP protein (A,C) or solely expressed (B,D). Scale bar = 10 μm.

3.B.2 Do HepG2 cells express multiple pancreatic phenotypes following ectopic expression of pCS2-TTR-XlhboxVp16?

3.B.2.1 Expression of pancreatic exocrine and endocrine markers

The pancreas is composed of multiple cell types, including exocrine and

endocrine cells (Percival and Slack, 1999). We used pancreatic tissue from E11.5 mouse embryos cultured for 7 days as control tissue for immunostaining of amylase, insulin, glucagon, somatostatin and pancreatic polypeptide (Figure 3.8). The question arises whether ectopic expression of the pCS2-TTR- Xlhbox8Vp16 plasmid simply induces pancreatic gene expression or whether it is possible to induce the formation of specific pancreatic cell types. Following transfection of HepG2 cells with the pCS2-TTR-Xlhbox8Vp16 plasmid, some cells expressed pancreatic exocrine amylase (Figure 3.9F) and endocrine hormones insulin (Figure 3.9G), glucagon (Figure 3.9H), and somatostatin were expressed (Figure 3.9I) in HepG2 cells by the fifth day, although the efficiency of conversion to pancreatic phenotype was extremely low. The only principal pancreatic hormone not observed was pancreatic polypeptide (PP, Figure 3.9J). This observation may be explained because of the small proportion of PP cells in the normal pancreatic islets (~ less than 1% of total endocrine cells (Slack, 1995)). In contrast, the HepG2 cells that were transfected with the control construct pCS2-TTR- Vp16 showed no pancreatic protein expression at all (Figure 3.9A-3.9E).

As well as protein detection by immunohistochemistry, we studied the expression of pancreatic genes by RT-PCR. This approach is complementary to immunodetection because it can show low levels of mRNA and, using the Light Cycler system, can provide quantitative results. The results showed that there is low-level expression of some, but not all, pancreatic genes, in the parental HepG2 cells under the same PCR conditions (28 cycles). The pancreatic genes include amylase and somatostatin, and an appreciable level of pancreatic polypeptide (Figure 3.10A). The real-time quantitative RT-PCR of cells transfected with pCS2-TTR-Xlhbox8Vp16 plasmid showed a sharp increase in mRNA expression for amylase, insulin, glucagon and somatostatin. The rise in mRNA expression occurred between 3 and 5 days after transfection, consistent with the timing of appearance of the pancreatic proteins

(Figure 3.10B-3.10E). It is noteworthy that pancreatic polypeptide mRNA was also upregulated on the 5th day after transfection (Figure 3.10F). The difference between RT-PCR and immunostaining results may be due to the limited sensitivity of protein detection by immunostaining (e.g. the protein may be folded so that the epitope is not apparent to the antibody). Altogether, these results demonstrate that the Xlhbox8Vp16 plasmid can cause HepG2 cells to express both exocrine and endocrine pancreatic genes.

Figure 3.8

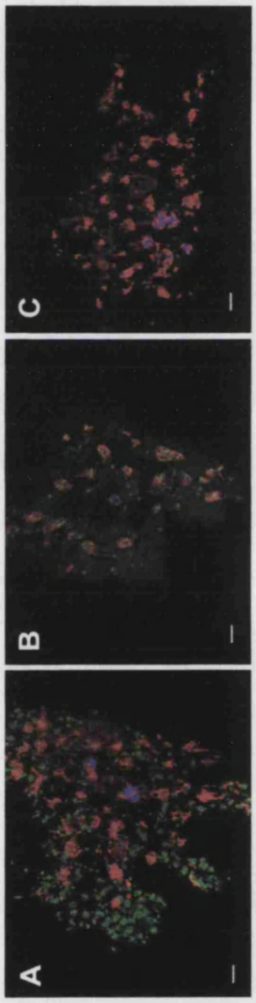


Figure 3.9

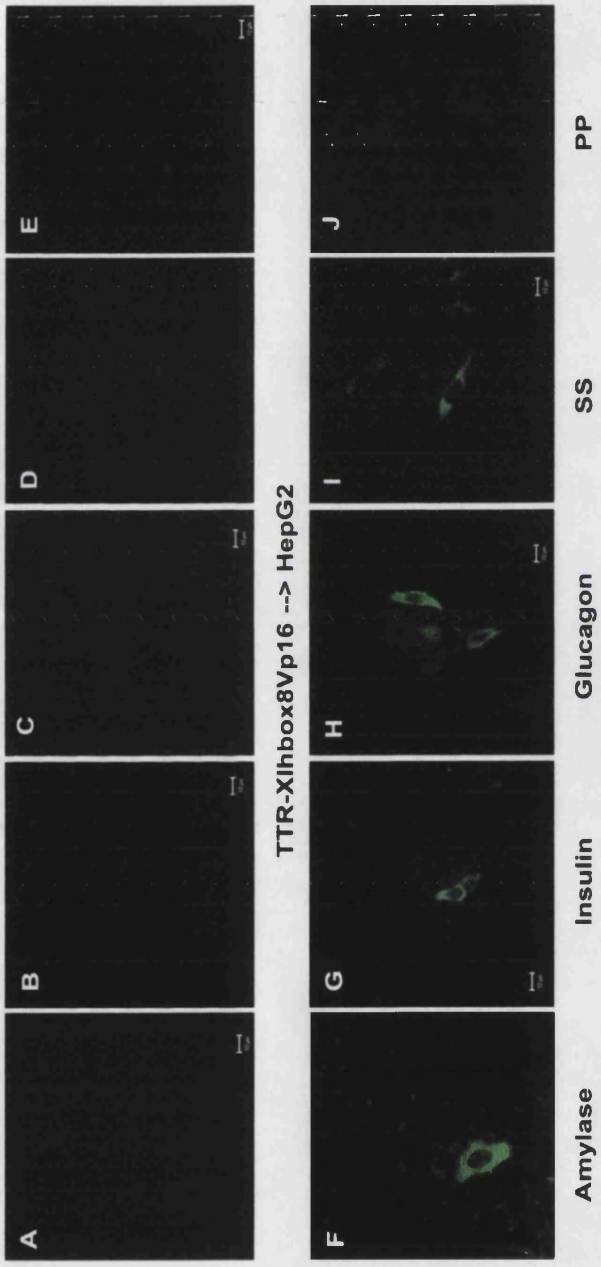


Figure 3.8 Pancreatic proteins expressed in cultured dorsal pancreatic buds. Amylase (A), somatostatin (B) and pancreatic polypeptide (C) are shown in green and insulin and glucagon are shown in red and blue respectively. Scale bar: 100 μm.

Figure 3.9 Pancreatic proteins are induced in HepG2 cells after transfection with pCS2-TTR-Xlhbox8Vp16 plasmid. Amylase (F), insulin (G), glucagon (H), somatostatin (I) but not pancreatic polypeptide (J) was detected at 5th day after transfection of pCS2-TTR-Xlhbox8Vp16. In contrast, no pancreatic protein expressing cells were observed in pCS2-TTR-Vp16 transfected cells (A-E). Scale bar: 10 μm.

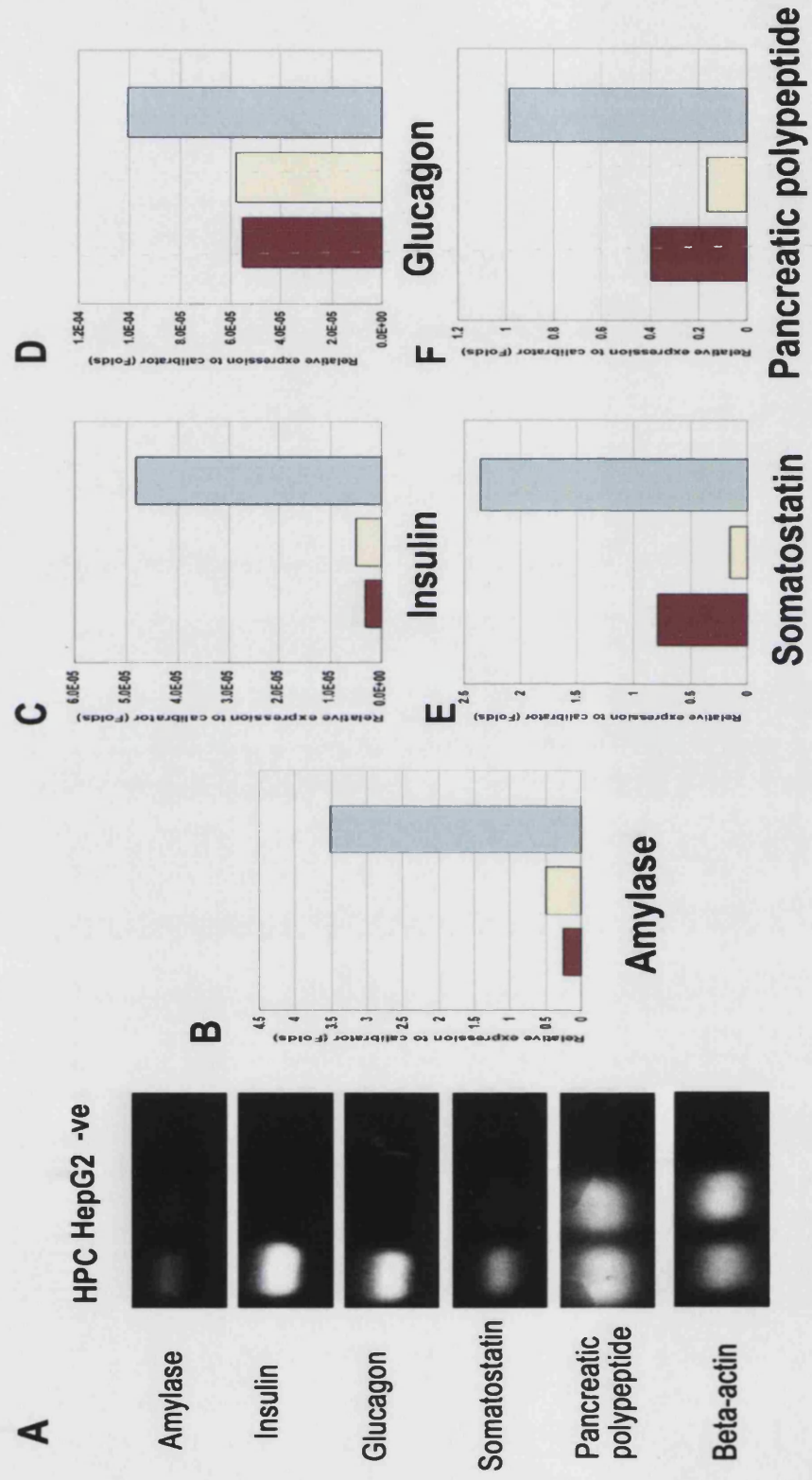

A
HPC HepG2 -ve
Amylase
Insulin
Glucagon
Somatostatin
Pancreatic polypeptide
Beta-actin
B
Amylase
C
Insulin
D
Glucagon
E
Somatostatin
F
Pancreatic polypeptide
Relative expression to calibrator (Folds)

Figure 3.10 Pancreatic mRNA expression is induced or upregulated in transdifferentiated cells. (A) Qualitative detection of pancreatic mRNAs in human pancreatic cells and in untransfected HepG2 cells. HPC: human pancreatic cells, -ve: no template control. (B-F) The relative mRNA expression was determined via real-time RT-PCR for amylase (B), insulin (C), glucagon(D), somatostatin (E) and pancreatic polypeptide (F) on 1st (purple bar) 3rd (yellow bar) and 5th day (light green bar) after transfection with pCS2-TTR-Xlhbox8Vp16. HPC=human pancreatic cells; -ve=no template control.

3.B.2.2 Colocalisation of pancreatic proteins in converted cells

An important issue in the conversion of HepG2 cells to pancreatic cells is the fidelity of the pancreatic cell types. In postnatal endocrine cells only one hormone is expressed in a cell. To investigate if transdifferentiated pancreatic cells also possess this characteristic, we co-stained HepG2 cells transfected pCS2-TTR-Xlhbox8Vp16 plasmid for pairs of different pancreatic markers. The results showed that none of the pancreatic proteins, including amylase, insulin or glucagon, were co-expressed in the same transdifferentiated cell (Figure 3.11). This means the converted cells are more likely to be normal differentiated pancreatic cell types rather than liver cells that are ectopically expressing pancreatic genes.

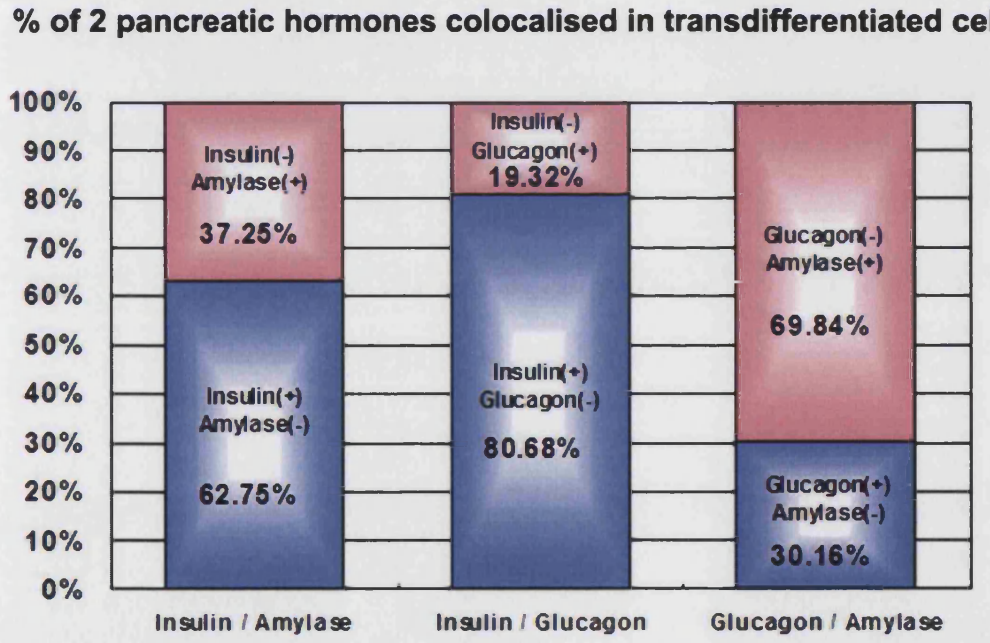


Figure 3.11 The histogram shows the number of pCS2-TTR-Xlhbox8Vp16 transfected cells stained for two pancreatic proteins. The results showed that no co-expressing cells were detected. Numbers of counted cells = 153, 88, 126 for insulin / amylase, insulin / glucagon and amylase / glucagon experiments, respectively.

3.B.3 Downregulation of hepatic proteins in converted HepG2 cells

3.B.3.1 Loss of hepatic proteins in transdifferentiated cells

It is possible that cells expressing pancreatic proteins continue to express liver genes. To examine the fate of the hepatic proteins in the cells expressing pancreatic proteins, dual-immunostaining for several hepatic proteins (e.g. albumin, α1 anti-trypsin, haptoglobin, transferrin and transthyretin), was performed along with the β-cell hormone insulin. This was performed in control (pCS2-TTR-Vp16) and in Xlhbox8Vp16 transfected HepG2 cells. The results indicated that the hepatic proteins in transformed cells were lost in some insulin-positive cells (Figure 3.12F, 3.12G and 3.12N) but were present in others (Figure 3.12H-3.12M, 3.12O). By contrast, the cells transfected with the control pCS2-TTR-Vp16 construct showed no insulin expression or loss of hepatic protein expression (Figure 3.12A-3.12E). The proportion of converted cells with or without hepatic markers was determined. Between one-sixth and a quarter of the insulin positive cells had completely lost hepatic proteins including albumin (25%), α1 anti-trypsin (24%) or transferrin (16%) and so are presumably transdifferentiated pancreatic β-like cells (Figure 3.13). We also examined the time-course of albumin expression in transfected cells. To quantify the number of albumin-expressing transfected cells we identified immunostained albumin and Vp16. A progressive loss of albumin over the 5-day period in Vp16 expressing cells was found (Figure 3.14). So although the majority of cells do still contain liver proteins at 5 days it is likely that albumin expression has been turned off and the protein is gradually decaying. Based on above data, the two main criteria for transdifferentiation characterisation of phenotype of the converted cells and

demonstration of the relationship between the original and converted cells were fulfilled to some extent. Therefore, it might be acceptable to use the term transdifferentiated cells for these pancreatic-like cells.

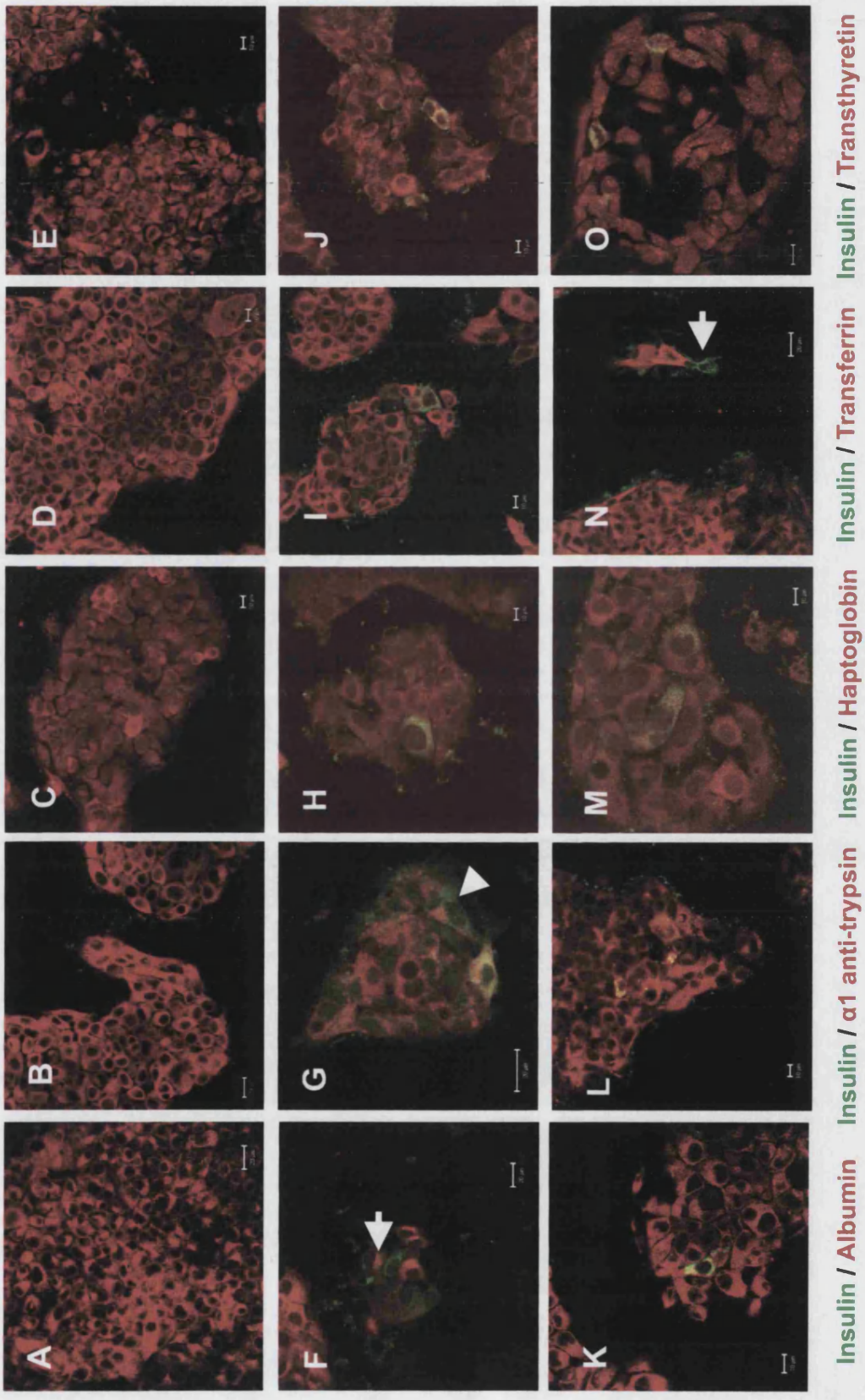

A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
Insulin / Albumin
Insulin / α1 anti-trypsin
Insulin / Haptoglobin
Insulin / Transferrin
Insulin / Transthyretin

Figure 3.12 Hepatic proteins were repressed in some transdifferentiated cells. HepG2 cells were transfected with control pCS2-TTR-VP16 (A-E) or pCS2-TTR-Xlhbox8Vp16 plasmids (F-O) and stained for the hepatic markers albumin (A,F,K), α_1-antitrypsin (B,G,L), haptoglobin (C,H,M), transferrin (D,I,N) and transthyretin (E,J,O) (red) and insulin (green). Insulin was not expressed in the pCS2-TTR-VP16 cells. The co-expression of insulin with a liver protein (H-M and O) is observed in some cases while some cells express insulin without the liver protein (F,G and N, white arrows). Scale bar: 10μm in C, D, E, H, I, J, K, L and M, 20μm in A, B, F, G, N and O.

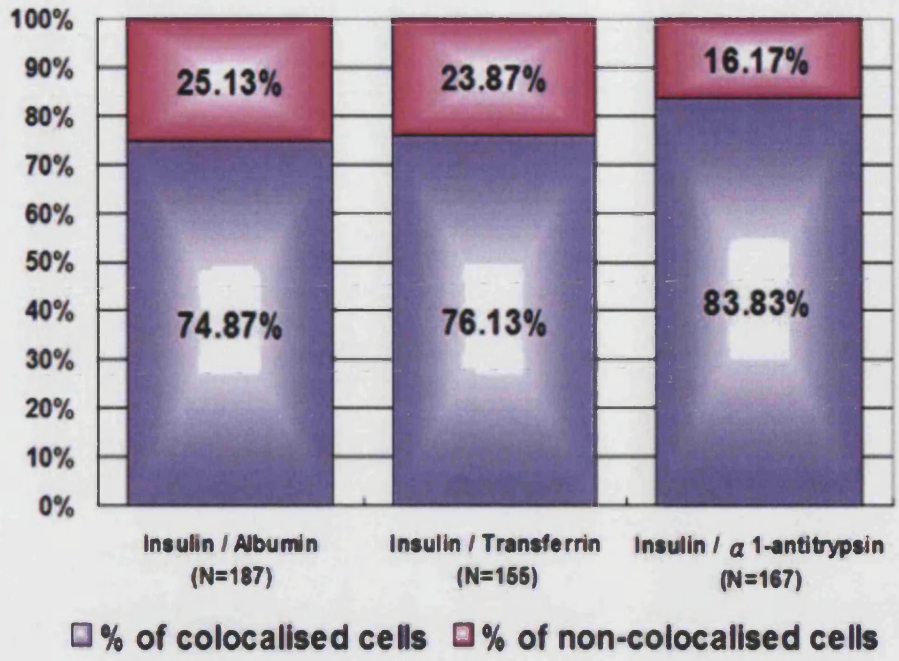


Figure 3.13 The percentage of cells co-expressing insulin and hepatic markers at day 5 after transfection of pCS2-TTR-Xlhbox8Vp16.

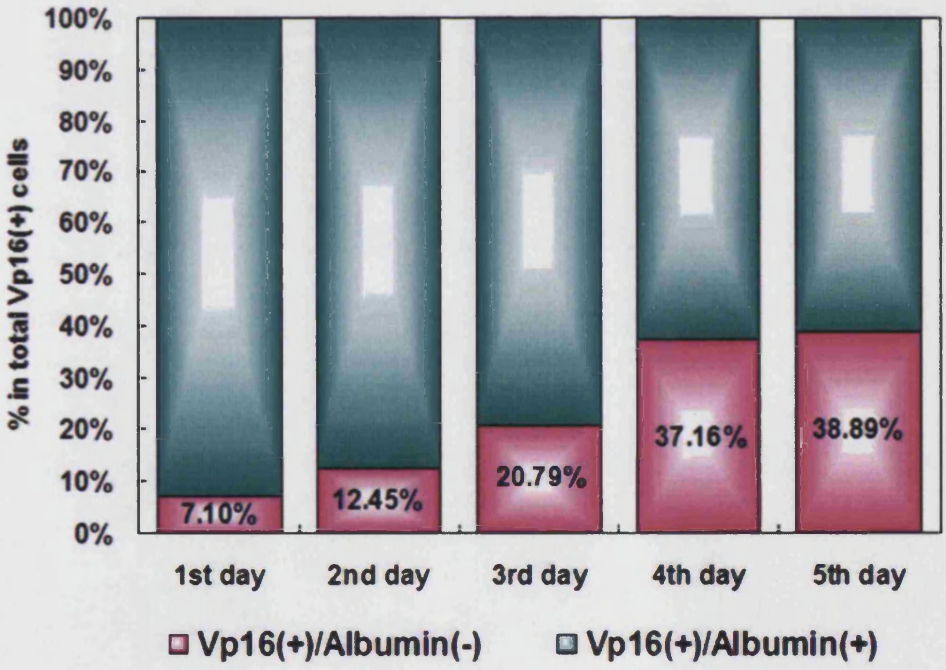


Figure 3.14 Progressive loss of albumin in pCS2-TTR-Xlhbox8Vp16 transfected HepG2 cells. These data were shown from the determination of 6 random fields of two independent experiments based on the immunofluorescent detection of Vp16 and albumin proteins.

3.B.3.2 Suppression of hepatic promoter activity in Xlhbox8Vp16 transfected cells

In order to further confirm the repression of the hepatic phenotype in transdifferentiated cells, another set of experiment was designed. We compared the promoter activity in cells transfected with the pCS2-TTR-Xlhbox8Vp16 construct and those transfected with either: (1) CMV- Xlhbox8Vp16 or (2) TTR-nGFP. In the first experiment, the CMV promoter is expected to be continuously active regardless of the state of differentiation of the cell. The proportion of Vp16 positive cells in the total cell population was measured on each day and then converted to a relative ratio for the TTR versus the CMV construct (Figure 3.15A). Although the percentage of Vp16 positive cells decreased in cells transfected with either construct, there was more repressive activity of TTR promoter in contrast to CMV promoter. A similar experimental procedure was carried out in TTR-Xlhbox8Vp16 versus TTR-nGFP transfected cells. The TTR-nGFP construct was used as a negative control for the effect of the transgene on the TTR promoter. The same measurement was performed and the result was presented as a proportion of Vp16 positive to nGFP positive cells. By contrast, the number of Vp16 expressing cells was significantly less than GFP positive cells at the same time point (Figure 3.15B). These experiments implied that TTR promoter activity is downregulated in TTR-Xlhbox8Vp16 transfected cells, in other words, the hepatic phenotype is switched off during transdifferentiation. These figures do not match Figure 3.4 quantitatively, but the precision of the results is limited by the fact that there is a certain loss of transfected cells during the culture period, and means that the results should be treated as qualitative rather than quantitative.

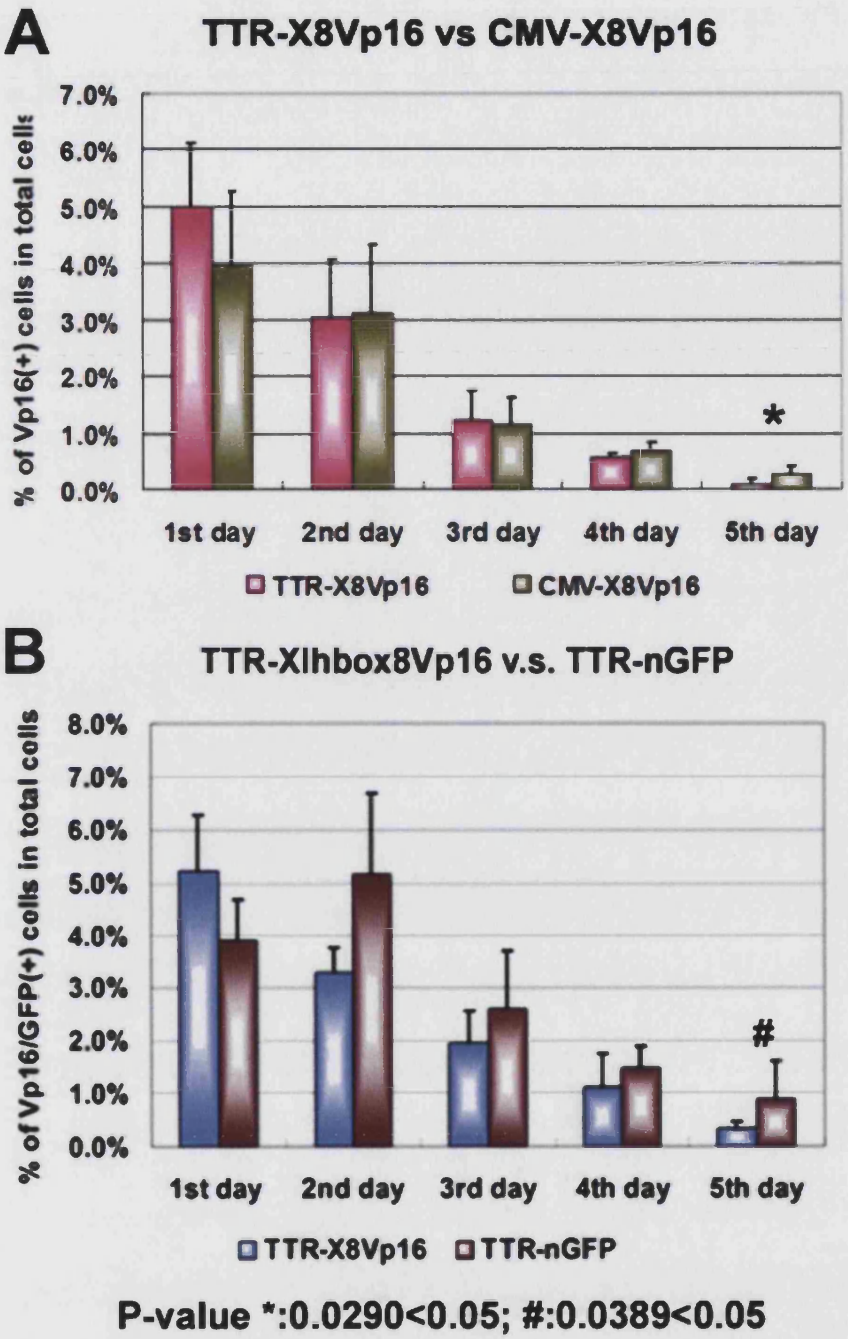


Figure 3.15 Repression of hepatic promoter activity in transdifferentiated cells. (A) Vp16 positive cells as a percentage of total cells in pCS2-TTR-Xlhbox8Vp16 or pCS2-CMV-Xlhbox8Vp16 transfected HepG2 cells. The different time points after transfection are shown. (B) Vp16/GFP positive cells as a percentage of total cells in pCS2-TTR-Xlhbox8Vp16 or pcDNA3-TTR-nGFP transfected HepG2 cells. The results on different days after transfection are shown.

3.B.4 The transgene is only transiently required to induce transdifferentiation

As mentioned previously, one possible clinical application for this study is to provide an alternative treatment for type 1 diabetes melliitus. Currently, insulin injection is the usual treatment for type 1 diabetic patients. However, regardless of the use of either traditional injection injections or the insulin pump to achieve the normoglycaemia in diabetic patients, the necessity of repeated injections or invasive therapy has seriously affected patients' quality of life. If gene therapy is ever to be of use, to avoid unexpected effects the shorter the time the transgene is expressed in the cells, the better. We followed the Xlhbox8 expression using both qualitative and quantitative real-time RT-PCR. The results showed a remarkable decrease of Xlhbox8 at the 5^{th} day following the transfection of pCS2-TTR-Xlhbox8Vp16 in contrast with levels after 1 day of transfection (Figure 3.16) and compared to the levels of insulin and amylase by day 5 (Fig. 3.10). This examination correlates with the TTR promoter experiments and suggested that the transgene for transdifferentiation of HepG2 cells to pancreatic cells is only transiently required, and that once the pancreatic fate is acquired it remains stable.

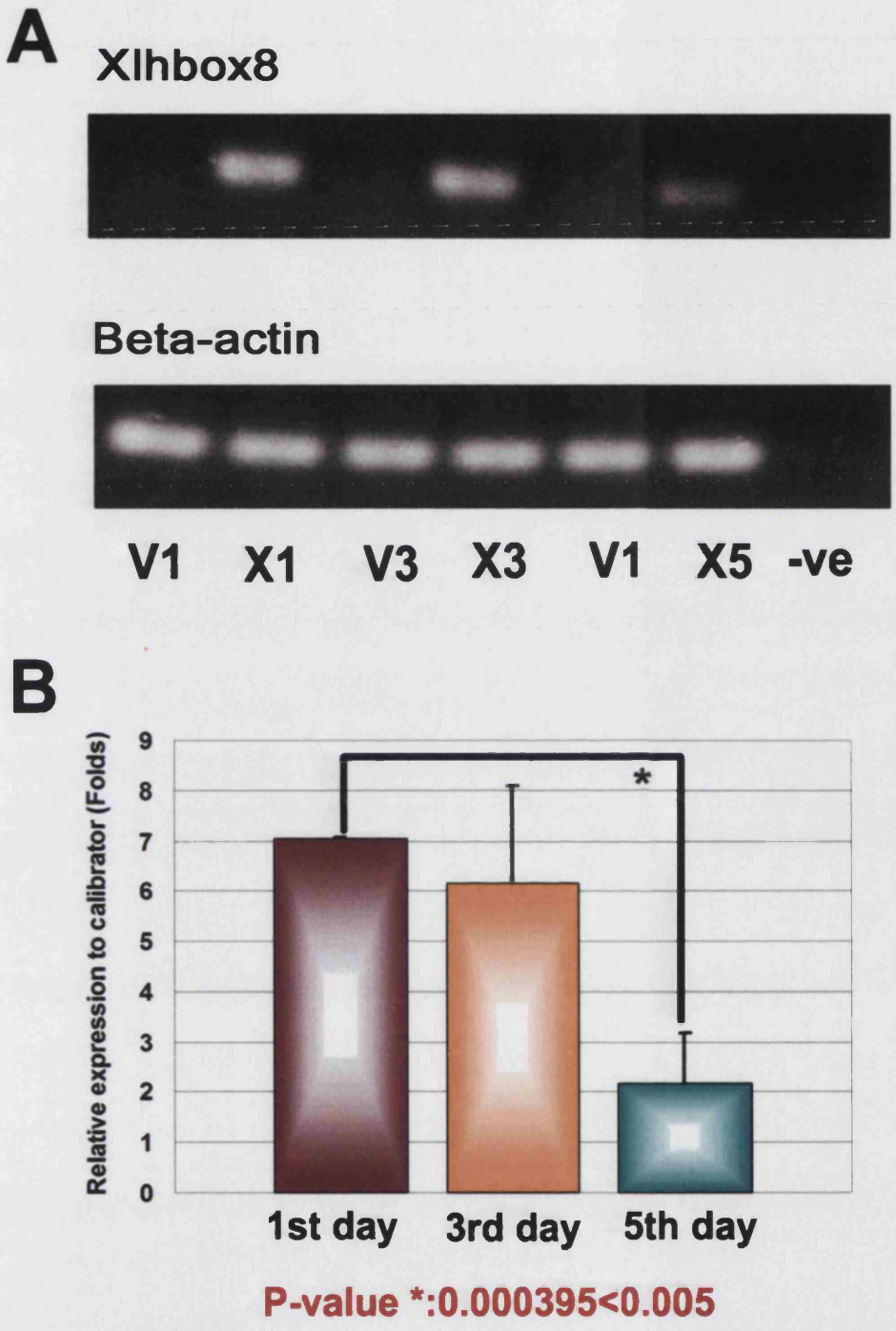


Figure 3.16 RT-PCR analysis for Xlhbox8 trasngene expression in transdifferentiated cells. (A) Qualitative RT-PCR showing decline of Xlhbox8 transcripts over the 5-day post-transfection period. V=pCS2-TTR-Vp16 control; X=pCS2-TTR-Xlhbox8Vp16 transfected cells. (B) Real-time quantitative RT-PCR showing downregulation of Xlhbox8 transcripts

3.C Origin of transdifferentiated cells

For the conversion of one cell type to another to be termed transdifferentiation, the relationship between the parental and transdifferentiated cells should be demonstrated. Although it has been demonstrated that a proportion of transdifferentiated cells expressed hepatic markers along with pancreatic protein insulin (Figure 3.12), it would be advantageous to develop a cell-tracing model to find out the origin of the transdifferentiated cells. Several experimental designs have used to determine the ancestor-descendant relationship. For example, it is possible to cross two animals containing a Cre recombinase regulated by tissue specific promoter (i.e. promoter X) and a certain reporter protein controlled by ubiquitous promoter (i.e. CMV promoter) to generate a offspring displaying the labelled X progenitors (Ghosh and Van Duyne, 2002; Sauer, 1998; Utomo et al., 1999). To examine the histological phenotype of *in vitro* cultures made from two individual strains of animals which have different distinguishable genotypes (such as Rosa 26 crosses with CD1 mice) was also demonstrated (Percival and Slack, 1999; Seymour et al., 2004). More recently, grafting of labelled, e.g. GFP-labelled, tissue to the same region of distinct animals was used to trail down the destiny of the specific cell types (Gargioli and Slack, 2004). Therefore, it was decided to generate a reliable reporter system in combination with immunostaining to address the question of the cell origin of the transdifferentiated cells.

3.C.1 Construction and expression property of CMV-Xlhbox8Vp16; CMV-DsRed plasmid

In order to trace the cell lineage using a transient transfection method, it is essential to have: (i) constitutively active promoter to drive transgene and reporter

gene and (ii) a reporter protein of low cytotoxicity.

Based on the steady-state levels of viral mRNA and the abundance of the protein product in CMV infected cells, the CMV promoter is one of the strongest promoters used in mammalian vectors (Thomsen et al., 1984). DsRed is derived from an Indo-Pacific sea anemone-relative, *Discosoma* species and can be highly expressed in mammalian cells (Jakobs et al., 2000; Matz et al., 1999). The total number of DsRed positive cells did not show any decay at least until 4 days after transfection of CMV-DsRed into HepG2 cells (Figure 3.17). Hence, DsRed is suitable for tracing the cell lineage of the transfected cells.

By combining CMV-Xlhbox8Vp16 and CMV-DsRed, a new construct was generated (Figure 3.18A). To understand the properties of this new construct, we determine the time-course of transgene and reporter expression by dual-staining for Vp16 and DsRed respectively. The results indicate that almost 80% of transfected cells co-expressed the transgene and DsRed even 5 days after transfection (Figure 3.18 B and C). This result suggested that both the Xlhbox8Vp16 and the DsRed were permanently expressed in all transfected cells regardless of phenotype. Although there are a few cells on day 1 that are negative for DsRed, this is probably due to the fact that the expression had not built up so that it was visible under fluorescence. In addition, there are a few more cells at each time point that are negative for Vp16. This may reflect a certain level of excision of the Xlhbox8Vp16 caused by recombination between the two CMV promoters.

3.C.2 Pancreatic phenotype is expressed in Xlhbox8Vp16 transfected cells

Five days after the introduction of the CMV-Xlhbox8Vp16; CMV-DsRed construct

into the HepG2 cells, transfected cells were compared with control CMV-DsRed transfected cells (Figure 3.19A). Insulin-positive cells were detected in CMV-Xlhbox8Vp16; CMV-DsRed transfected cells in a similar fashion to the results obtained from the TTR-Xlhbox8Vp16 transfected HepG2 cells (Figure 3.19B). Moreover, about half of the insulin producing cells came from transfected cells based on the detection of insulin/DsRed co-expression at the 5^{th} day after transfection of the plasmid. This result confirmed the source of transdifferentiated cells (Figure 3.19C). It is true that some insulin producing cells do not express dsRed. This does raise the possibility that cells might transdifferentiate without containing the Xlhbox8Vp16 themselves, through some sort of cell interaction. But we cannot conclude much from this fraction as the cells might lose either of the genes by recombination between the CMV sequences. The double positives are therefore more significant than the single positives.

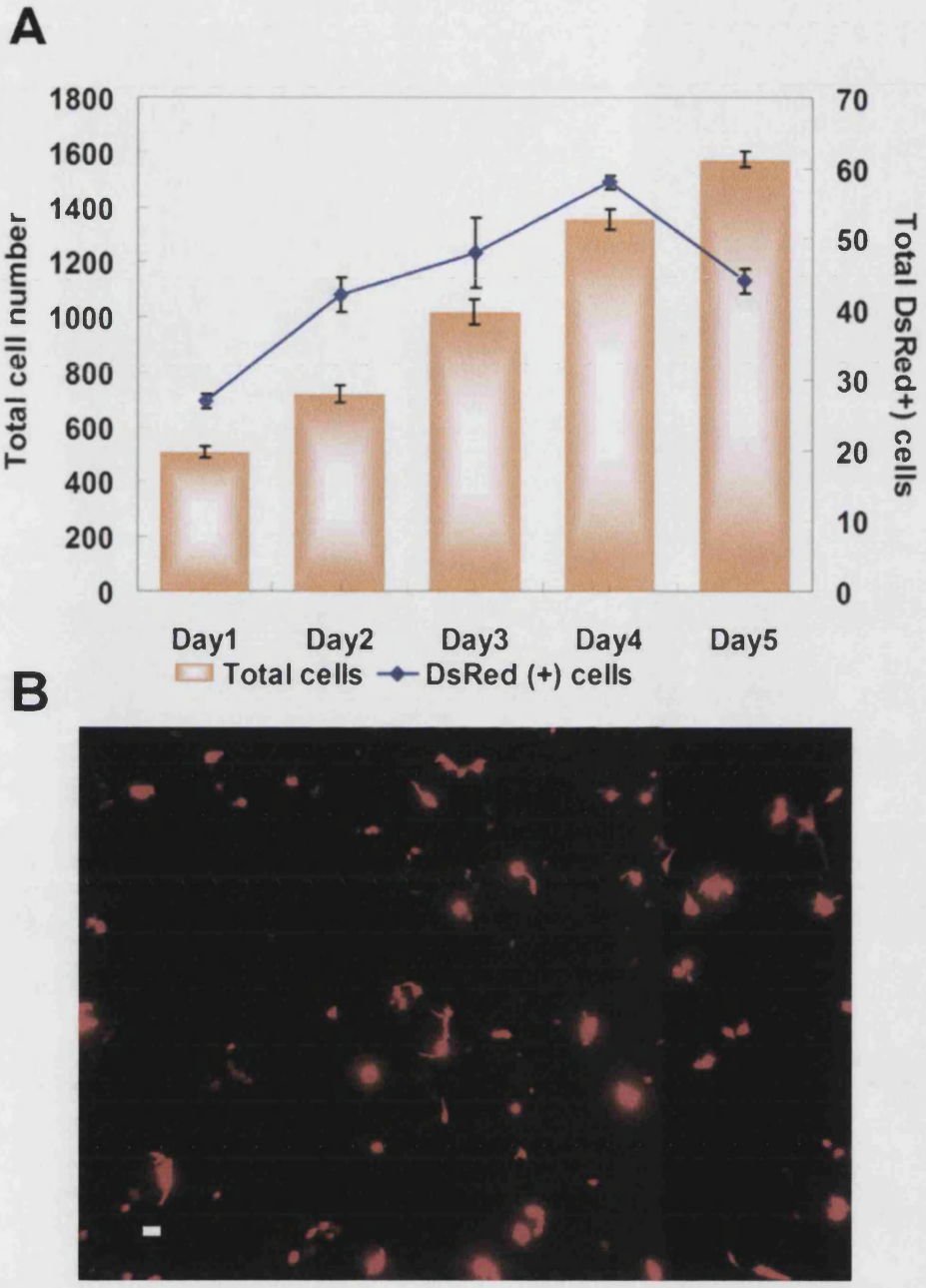


Figure 3.17 DsRed expression in HepG2cells transfected with pcDNA3-DsRed plasmid. 1μg pcDNA3-CMV-DsRed DNA was introduced into HepG2 cells and detected by cell counting from the 1st to the 5th day after transfection (A). Confocal microscope images (B) were taken on the 2nd day after transfection). It was shown that DsRed persists for at least 4 days and there is loss of expression after 5 days of transfection. Scale bar in B and C is 20 μm.

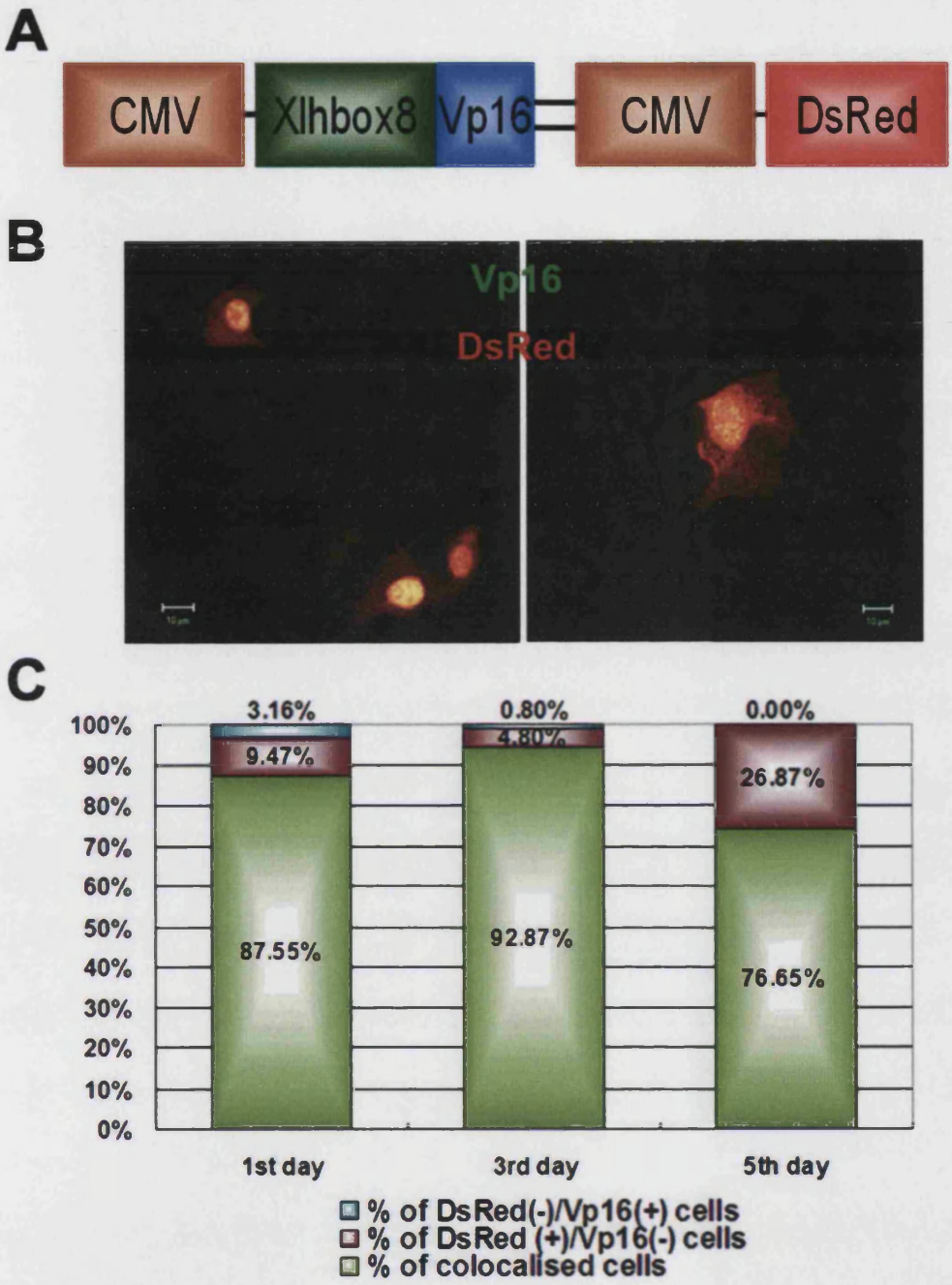


Figure 3.18 Testing of CMV-Xlhbox8Vp16; CMV-DsRed construct in HepG2 cells. (A) Schematic representation of CMV-Xlhbox8Vp16; CMV-DsRed construct; (B) The expression of Vp16 (green) and DsRed (red) for the 2-day CMV-Xlhbox8Vp16; CMV-DsRed transfected HepG2 cells. (C) Analysis of Vp16 and DsRed expression in HepG2 cells transfected with CMV-Xlhbox8Vp16; CMV-DsRed on the 1st, 3rd and 5th day after transfection. The graph was shown based on the data from the determination of 6 random fields from two independent experiments.

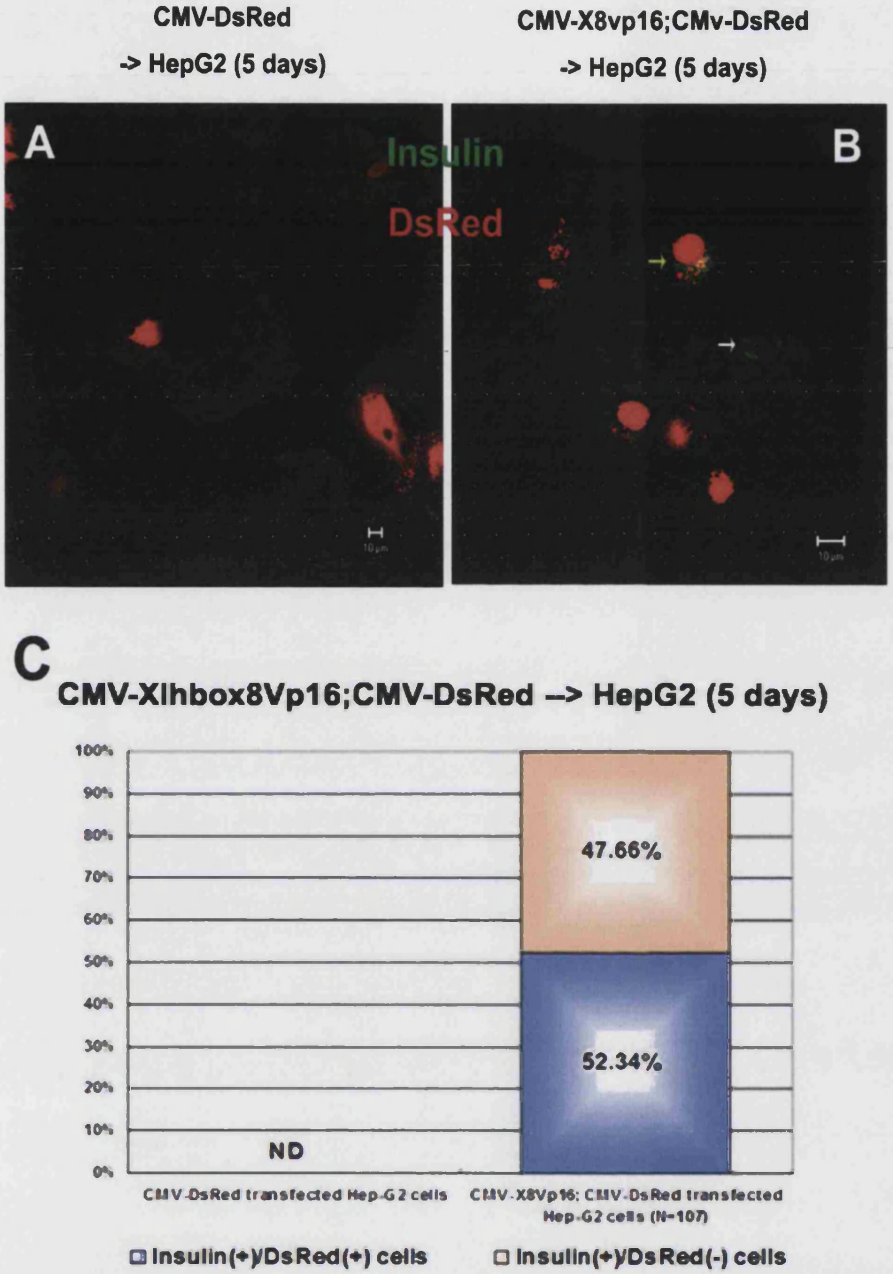


Figure 3.19 Cell origins of transdifferentiated cells. The origin of transdifferentiated cell was investigated in CMV-DsRed (A) and CMV-Xlhbox8Vp16; CMV-DsRed (B) transfected HepG2cells. Insulin (in green) positive cells were detected with (yellow arrow) or without (white arrow) co-expression with DsRed. (C) ratio of the DsRed(+)/Insulin(+) or DsRed(-)/Insulin(+) cells to total insulin (+) cells 5-days after transfection with CMV-Xlhbox8Vp16; CMV-DsRed plasmid. The figure was shown based on the data from the determination of 5 random fields of two independent experiments.

3.D Mechanisms to induce transdifferentiation

3.D.1 Vp16 does not work by promoting nuclear localisation

Previous work has shown that the Vp16 domain is essential to achieve transdifferentiation in the *Xenopus* system (Grapin-Botton et al., 2001; Heller et al., 1998; Horb et al., 2003). The Vp16 activation domain is a herpes virus protein and was shown to be a potent activator of transcription that is bound to the promoter region as a consequence of its association of several DNA-binding proteins, for example transcription factor TFIIH (Jiang et al., 1994; Xiao et al., 1994). Several mechanisms have been proposed to account for the activity of the Vp16 domain. For example, it could be through the neutralisation of the repressive effect of histones on transcription or grouping of the factors involved in the initiation or activation of transcription regulatory factors. These factors include TATA-binding protein (TBP), TBP-associated components including transcription factor IID (TFIID) and transcription factor IIB (TFIIB) and histone acetyltransferase(Croston et al., 1992; Horikoshi et al., 1988; Ikeda et al., 2002; Lin and Green, 1991). Therefore, it is possible that part of the activity of Vp16 domain may depend on increasing nuclear localisation of the Xlhbox8 protein. To investigate this hypothesis, two more plasmids were generated: CMV promoter driving either mouse Pdx1 or mouse Pdx1Vp16 fusion protein. These experiments were done with the mouse sequence rather than the *Xenopus* one because of the limited availability of a suitable antibody to detect the *Xenopus* protein. These two constructs were then transfected into HepG2 or Panc-1

cells to investigate the intracellular localisation of Pdx1. Panc-1 is a human pancreatic ductal cell line derived from 56-year-old caucasian male and does not express Pdx1. Panc-1 provides a control comparison to understand whether the transcriptional activation effect of the Vp16 sequence is only exerted in liver; and, more specifically, whether or not the localisation of Pdx1, before and after tagging with Vp16 domain, is only altered in "pancreatic-like" cells.

The rat insulinoma cell line, RIN-m5F, was used as a control for the Pdx1 antibody activity since endogenous Pdx1 is highly expressed in this cell line (Figure 3.20A, 3.20B). Pdx1 is expressed either in the nucleus or the cytoplasm in both HepG2 and Panc-1 cells (Figure 3.20C-3.20F). There was no significant difference in nuclear localisation between Pdx1 and Pdx1Vp16 in either cell line by quantitative cell counting at 5th day after transfection. The proportion of nuclear Pdx1 or Pdx1Vp16 in both cell lines was estimated at 65%~75% of total transfected cells (Figure 3.21). In conclusion, Vp16 does not exert its effect by increasing nuclear localisation.

HepG2 cells were transfected with either CMV-Pdx1, CMV-Pdx1Vp16 or TTR-Xlhbox8Vp16 to determine whether there were any difference in the nuclear localisation. Depending on the construct, the nuclear localisation of the transgene was examined by detection of Vp16 or Pdx1. A significant increase in nuclear localisation of transcription factors was observed in TTR- Xlhbox8vp16 transfected cells (Figure 3.22). This may result from a sequence difference between the mouse and *Xenopus* genes. Therefore, by using the *Clustal X* alignment software and the domain tool on the PubMed website, the amino acid sequences of mouse Pdx1 and *Xenopus* homologue Xlhbox8 were compared. Although 100% identity of input sequences (human, mouse and *Xenopus* Pdx1 homologue) from E151 to R216 (the homeobox region of Pdx1 gene) was displayed, the homology of total amino acid sequences in mouse and *Xenopus* was far different, about 58% identity (165 out of 283 amino acids)

(Figure 3.23). Based on this observation, we assumed that the difference of percentage of nuclear Xlhbox8 and mouse Pdx1 in transfected HepG2 cells might be attributed to the structural variance of Xlhbox8Vp16 and Pdx1Vp16.

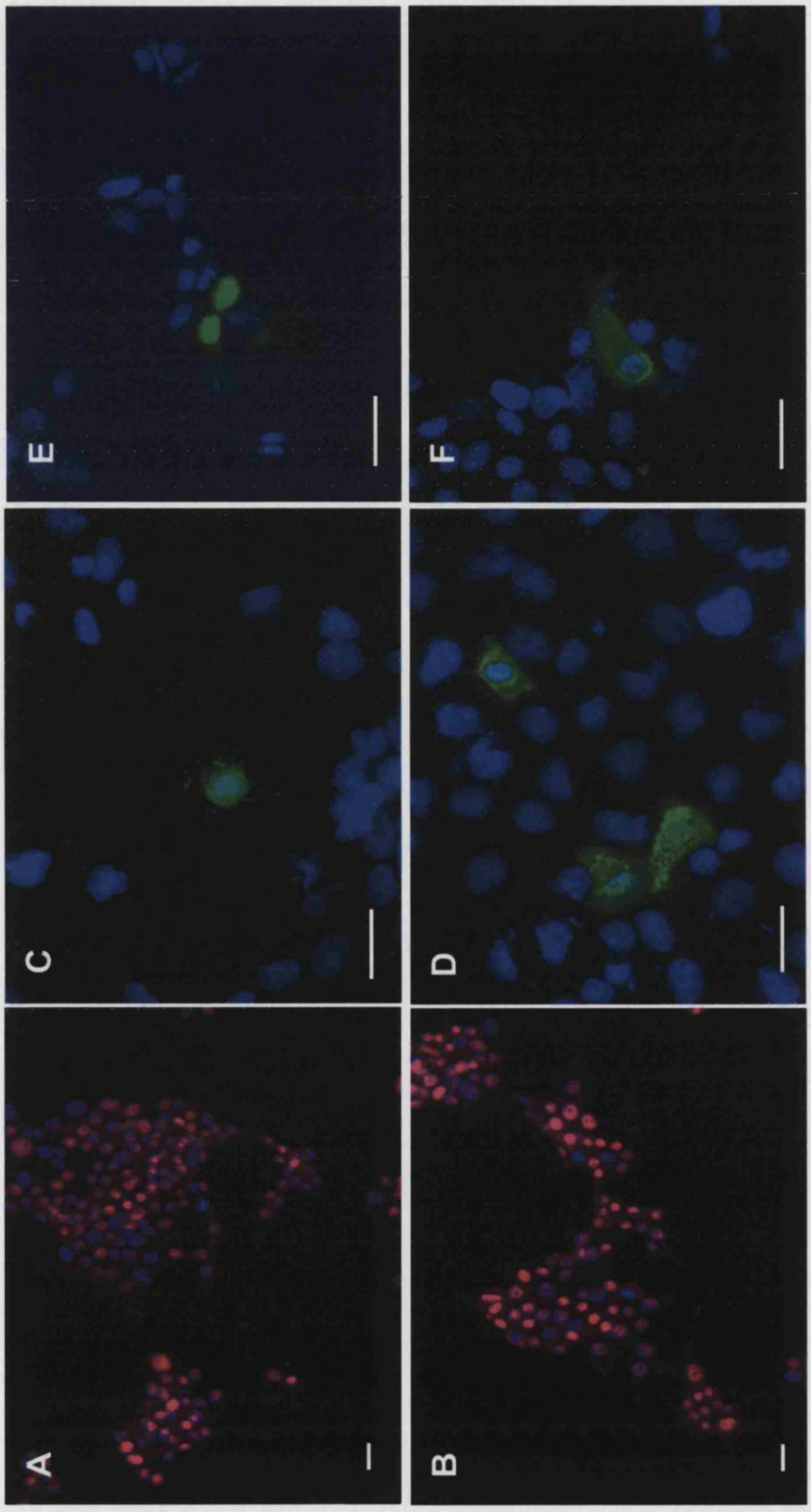


Figure 3.20

Figure 3.20 Nuclear localisation of Pdx1 or Pdx1Vp16 in different cells. Rat insulinoma (RIN-5F) cells (A,B), HepG2 cells (C,D) and human pancreatic ductal Panc-1 cells (E,F) immunostained for Pdx-1 (red in A,B; green in C,E) or Vp16 (green in D,F) and DAPI (blue) to determine transgene localisation. HepG2 and Panc-1 cells were transfected with pcDNA3-mpdx1 (C,E) or pcDNA3-mpdx1Vp16 (D,F) plasmid. Scale bar: 50 μm.

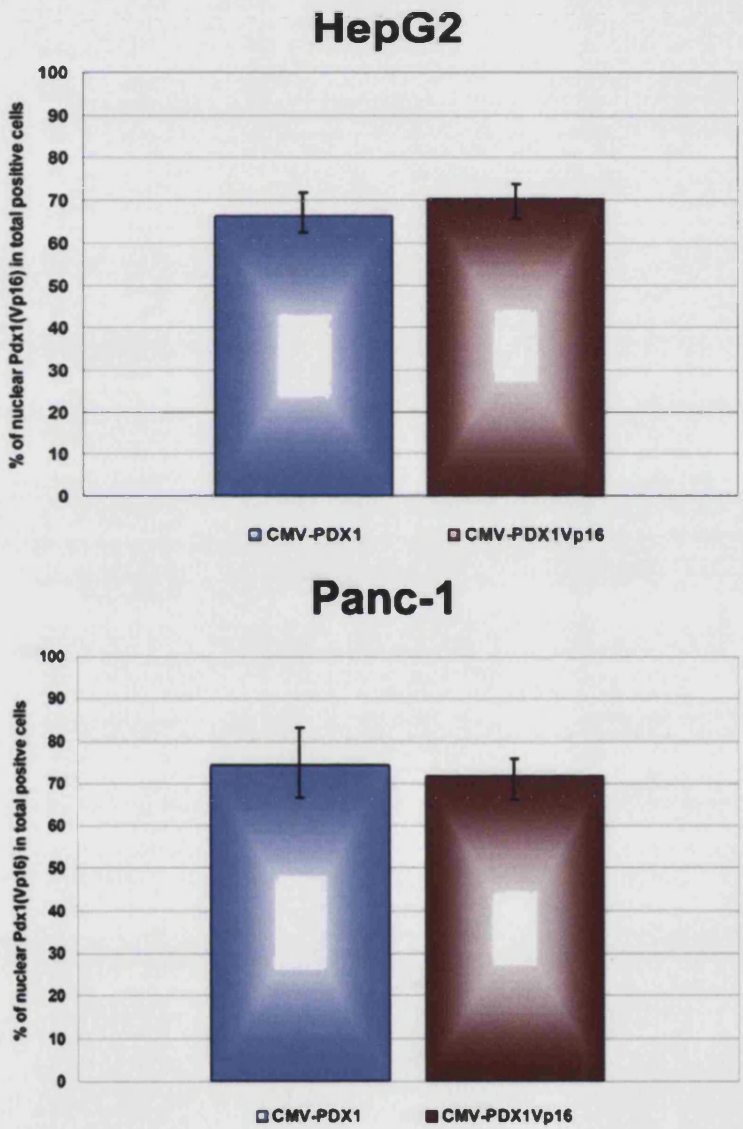


Figure 3.21 Pdx-1 nuclear localisation is not altered by the presence of the Vp16 activation domain. Cell counts were performed for nuclear Pdx1 expression on cells transfected with either Pdx1 or Pdx1Vp16 in HepG2 or Panc-1 cells. There was no significant difference between the absence and presence of Vp16.

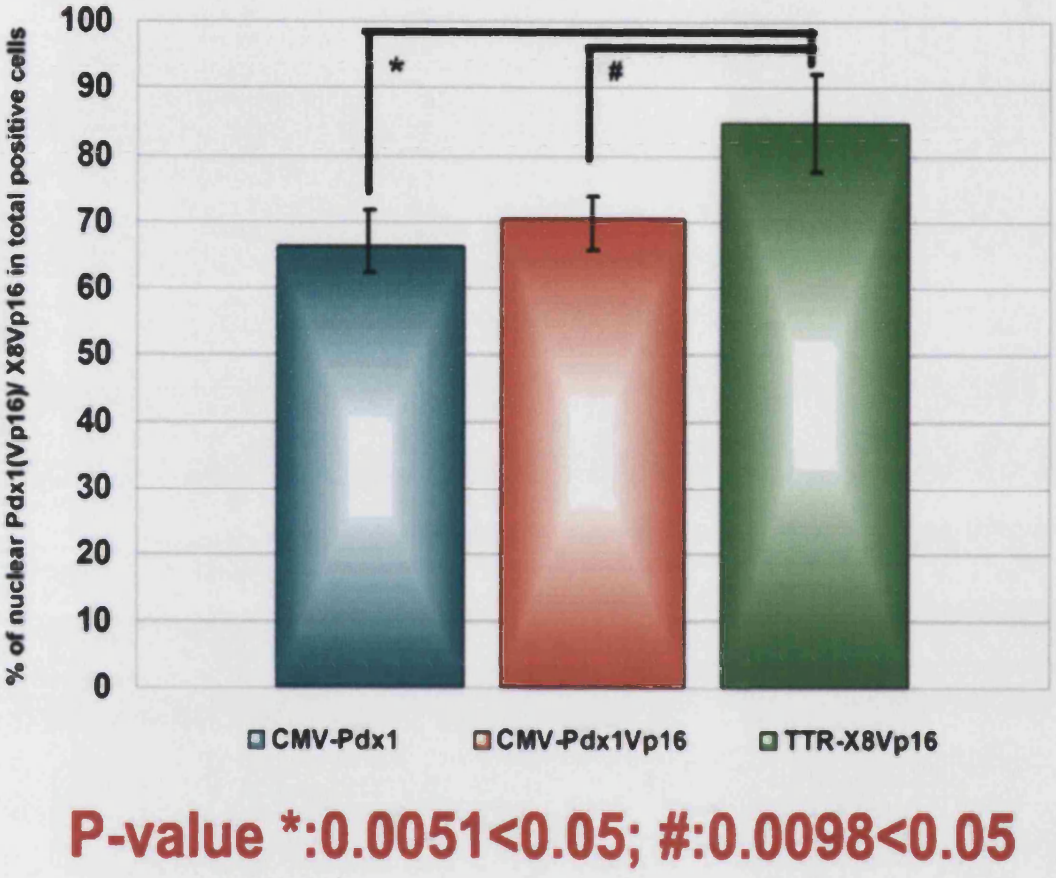


Figure 3.22 Variation of nuclear localisation of Pdx1, Pdx1Vp16 and Xlhbox8Vp16 in HepG2 cells. The graph represents the mean±S.D. for at least 5 random fields of 3 different slides.

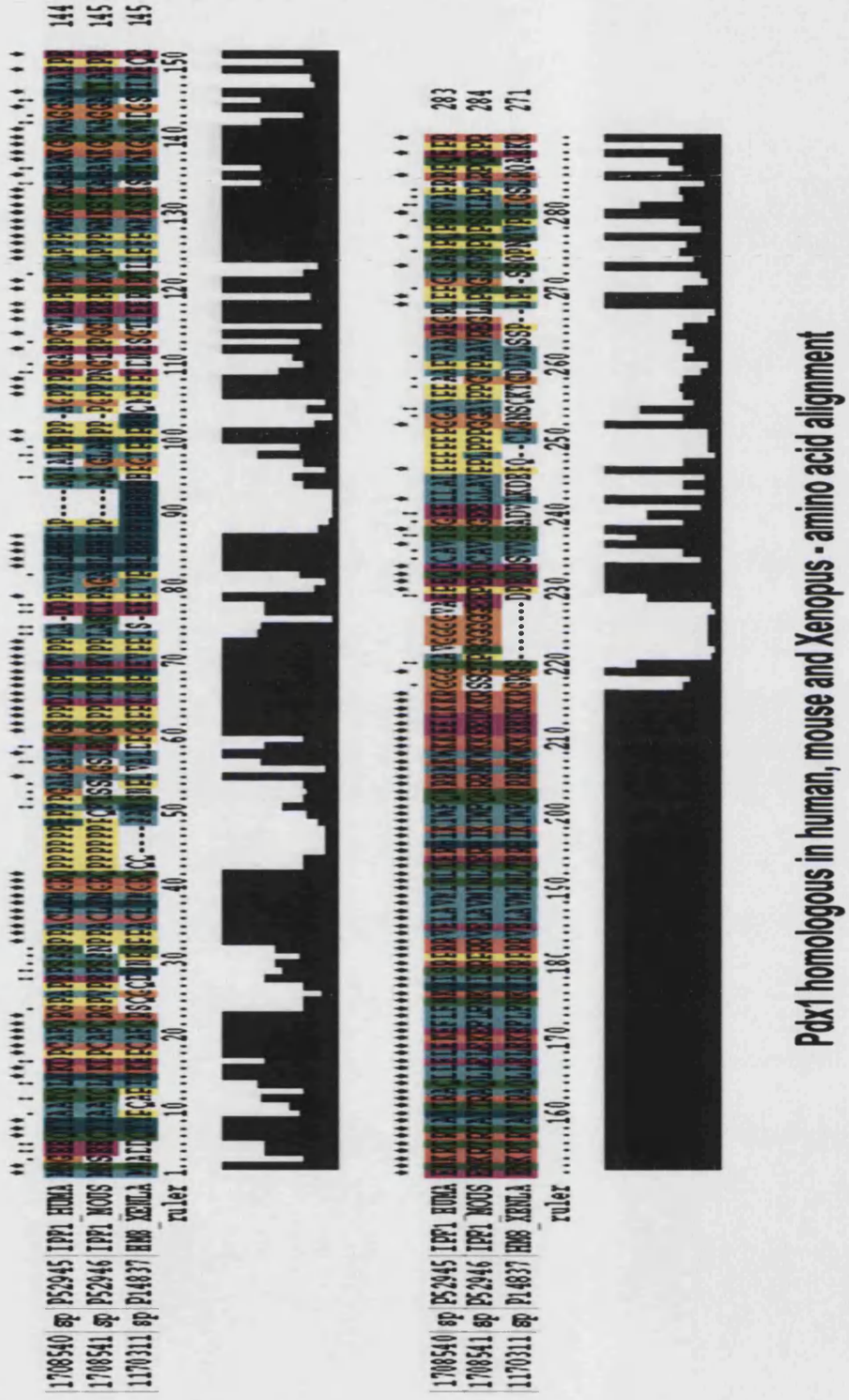


Figure 3.23 The alignment of human, mouse and Xenopus Pdx1 homologues using Clastal X software.

3.D.2 Higher glucose concentration does not increase nuclear Pdx1 in HepG2 & Panc-1 cells but increases insulin production in transdifferentiated cells

It has recently been shown that a high glucose environment stimulates nuclear translocation of Pdx1 from cytoplasm into nucleus (Macfarlane et al., 1999; Rafiq et al., 2000; Rafiq et al., 1998). This effect may mediate the increasing phosphorylation of Pdx1 by activation of phosphatidylinositol 3-kinase, SAPK2/p38 signalling pathways or directly by interacting with the nuclear import receptor family member, importin β1 (Elrick and Docherty, 2001; Guillemain et al., 2004). Using the idea that high glucose can promote insulin expression, it might also be possible to promote either the efficiency of transdifferentiation or maturation of transdifferentiated β-cells. To test this idea, the ratio of nuclear Pdx1 in CMV-Pdx1 transfected HepG2 and Panc-1 cells were compared in physiological (5.5mM) and high (25mM) glucose. The percentage of cells displaying nuclear Pdx1 showed no significant difference between these two culture conditions in either cell lines (Figure 3.24).

The *in vivo* data from Cao et al. suggested that high glucose treatment with mice injected with Pdx1Vp16 adenoviral construct improved the maturation of transdifferentiated cells (Cao et al., 2004). To further test if a higher concentration of glucose also enhanced the maturation of transdifferentiated cells in the HepG2 system, the cells were transfected with TTR-Xlhbox8Vp16 and incubated in high glucose (25mM) culture medium for either (1) an additional 2-8 hours after 5 days of transfection or (2) for the entire time (1-5 days) after transfection. The insulin and glucagon mRNAs were detected using real-time RT-PCR. The short-term incubation

showed the insulin mRNA was largely increased by about 10^4 folds after 6-hours treatment of high concentration glucose in comparison with the insulin expression at zero time point (Figure 3.25). Long-term exposure to high concentration glucose in HepG2 cells transfected with TTR-Xlhbox8Vp16 indicated that insulin and glucagon mRNA were significantly enhanced after prolonged high glucose treatment after 5 days (Figure 3.26). In summary, it can be concluded that high glucose promotes complete transdifferentiation in HepG2 cells but it may not be due to increased nuclear translocation of Pdx1. This work was done late in the thesis period so the result could not easily be exploited. However it suggests that many of the other results would have been much clearer had the experiments been performed in high glucose.

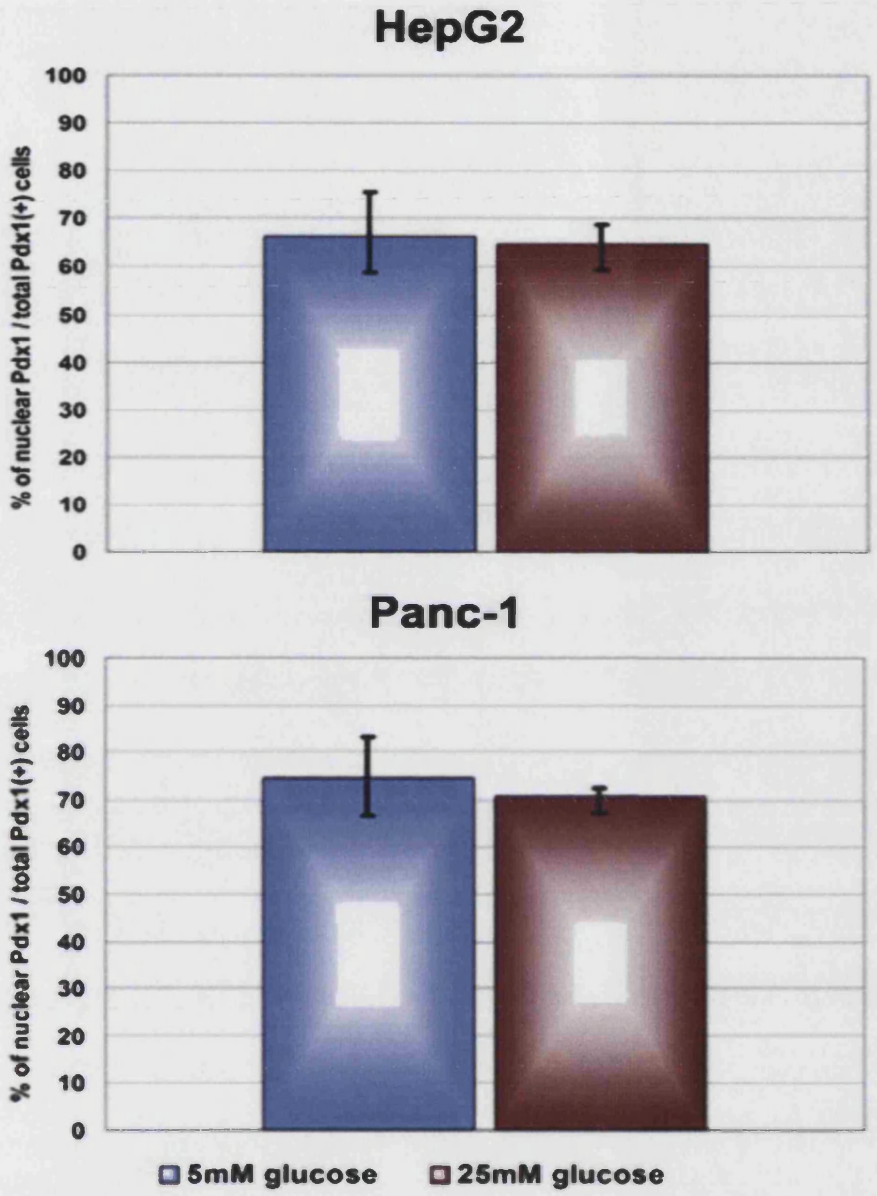


Figure 3.24 High glucose does not alter the nuclear localisation of Pdx1 in HepG2 and Panc-1 cells. Cell counting results showed no significant difference of nuclear Pdx1 expression between the cells incubated in medium with either 5.5mM or 25mM glucose for HepG2 and Panc-1 cells.

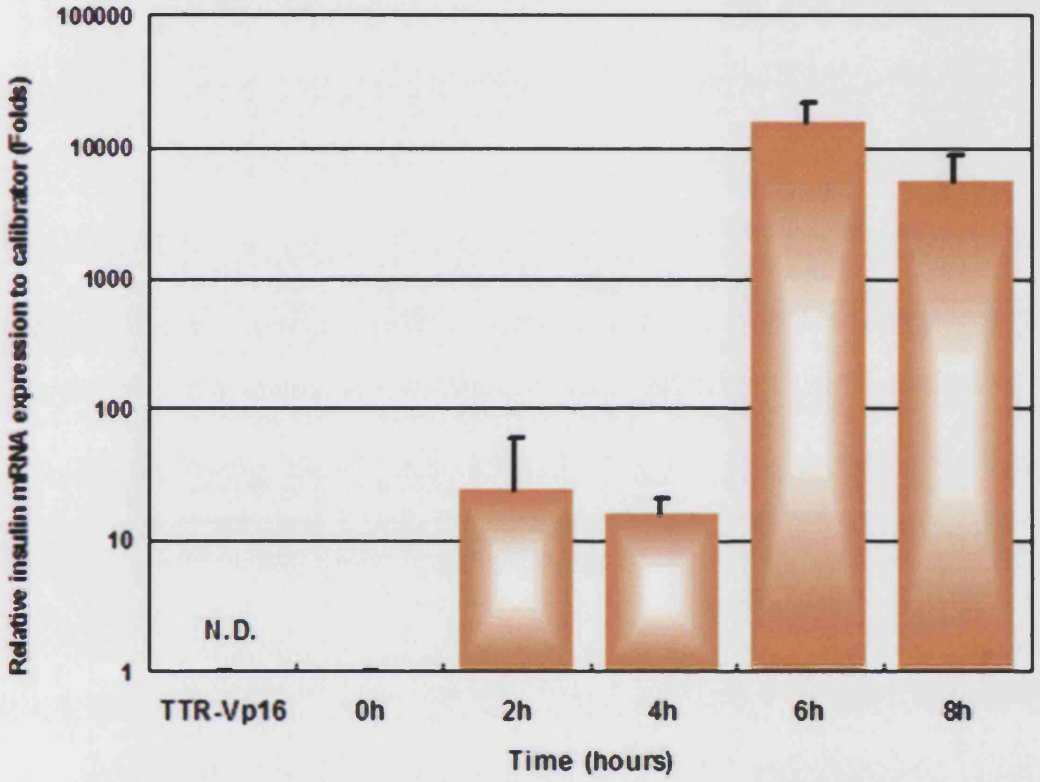


Figure 3.25 Short-term effect of high glucose on insulin mRNA in transdifferentiated cells. The cells were transfected with control (pCS2-TTR-Vp16) or pCS2-TTR-Xlhbox8vp16 plasmids and then were cultured in medium containing 5.5 mM glucose for 5 days and changed to the medium contained 25mM glucose for up to 8 hours. The real-time RT-PCR to detect insulin mRNA was then performed. The sharpest increase of the insulin occurred between 4 and 6 hours after incubation of transdifferentiated cells in high glucose. The result was presented as mean+S.D. for 3 different experiments.

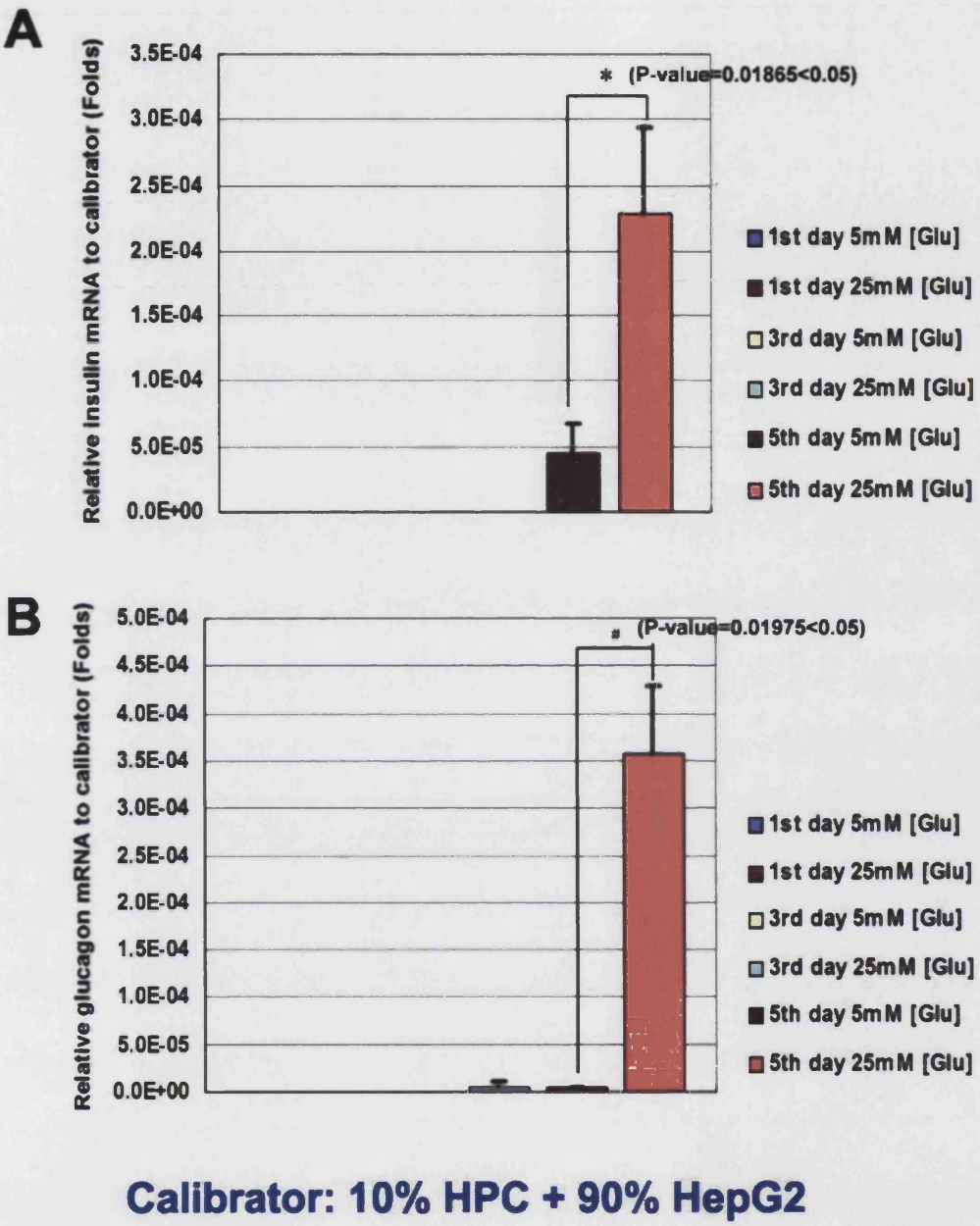


Figure 3.26 Long-term effect of high glucose on insulin mRNA in transdifferentiated cells. The cells were treated with normal or high glucose from the day after transfection of pCS2-TTR-Xlhbox8Vp16. The (A) insulin and (B) glucagon mRNA was then detected using real-time RT-PCR. The results are presented as mean+S.D..

3.E Endogenous pancreatic programme activation and division of transdifferentiated cells

Regarding the future possible clinical application of transdifferentiated cells for the treatment of diabetes, two interesting questions remained. First, is the endogeous pancreatic developmental programme induced in the course of transdifferentiation? Second, can the transdifferentiated cells proliferate? Previous work has shown that endogenous pancreatic transcription factors can be induced after exogenous genetic stimulation. Ber et al. infected mouse liver with the first generation adenoviral vector encoding rat *Pdx1.* Although the rat Pdx1 was infected, the mouse *Pdx1* gene was induced suggesting a self-feedback regulation (Ber et al., 2003). In the present study, the *Xenopus* Pdx1 homologue Xlhbox8 was used as the experimental material in a human cell line, it is therefore possible to design specific primers to detect human Pdx1 expression. Because of the low transdifferentiation efficiency, the culture period after transfection was extended up to 7 days and the PCR program was increased to 40 cycles to enrich the signals (Figure 3.27). This shows a low but reproducibly detectable signal from the endogenous human *Pdx1* gene in the transfected cells. This result suggests that Xlhbox8Vp16 transfected HepG2 cells do induce their endogenous Pdx1 gene.

The restricted availability of transplant pancreas has been an impediment for the treatment for type I diabetic patients. If cell transplantation is to be of use, it will be of benefit if the transdifferentiated cells can be expanded *in vitro.* To examine this issue, the detection of a mitotic marker, phosphohistone H3 (Ajiro et al., 1996), in transdifferentiated cells was performed. Some insulin producing cells were also positive for phospho H3 (Figure 3.28) suggesting that the transdifferentiated cells are capable of dividing.

Figure 3.27

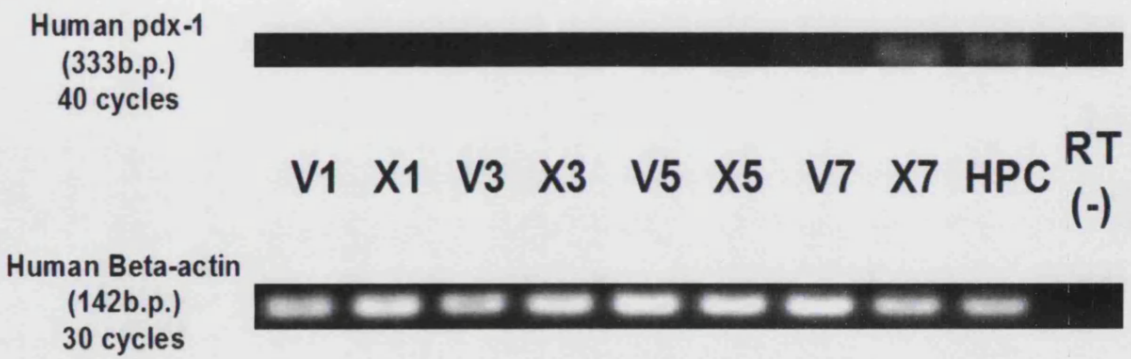


Figure 3.28

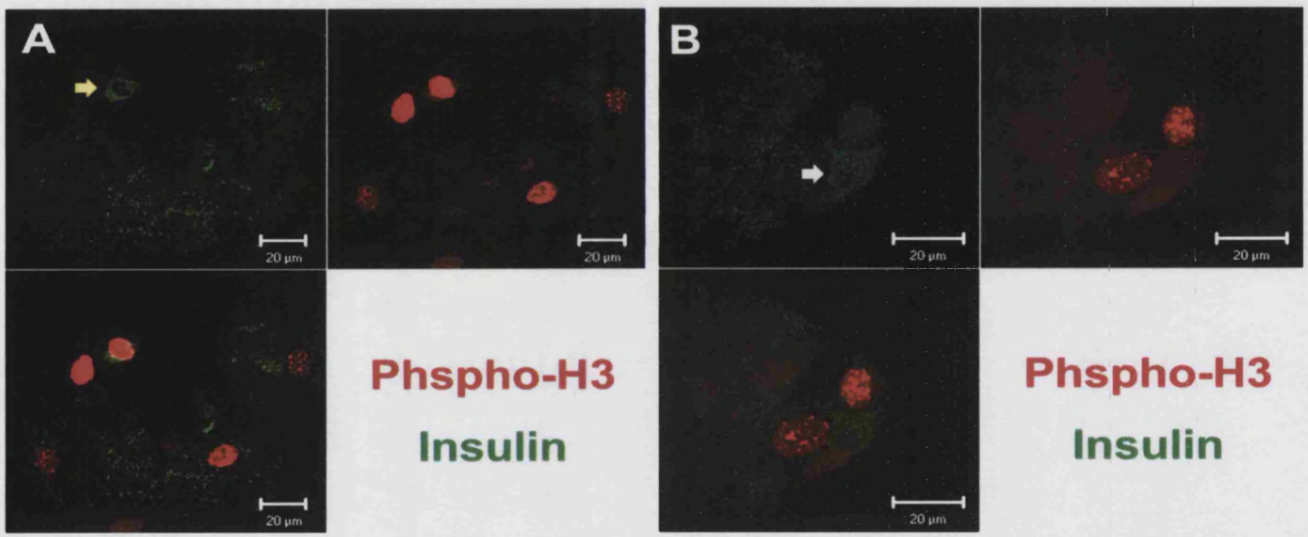


Figure 3.27 Endogenous Pdx1 expression in transdifferentiated cells. X=transfected with pCS2-TTR-Xlhbox8Vp16; V=controls transfected with pCS2-TTR-VP16, HPC=human pancreatic cells; -ve=no template.

Figure 3.28 Cell division in transdifferentiated cells. The 5-day pCS2-TTR-Xlhbox8vp16 transfected HepG2 cells were stained with insulin (green) and phosphohistone H3 (red, nuclear staining). The result shown that the insulin positive cells co-expressed with (yellow arrow in A) or without (white arrow in B) phosphohistone H3. Scale bar= 20 μm.

3.F Discussion

In the present chapter, the results show that transfection of Xlhbox8Vp16 into HepG2 cells is able to induce pancreatic cell phenotypes. The phenotypes include amylase-expressing exocrine cells and a range of endocrine cells, at least α (glucagon-expressing), β (insulin-expressing) and δ (somatostatin-expressing) types (Figure 3.9 and 3.10). It is noteworthy that a similar pancreatic cell distribution of the transdifferentiated cells to normal pancreatic cells was present. In normal pancreas, β-cells constitute the major endocrine cell type, followed by α-cells, ghrelin-cells, δ-cells and finally PP-cells make the smallest number (Slack, 1995). Among the transdifferentiated cells insulin-expressing cells are about four times more prevalent than glucagon-producing cells under the same culture conditions (Figure 3.11). One interesting result displayed from this work is the existence of the "dynamic" transdifferentiation process. This statement is based on the observation of both insulin / hepatic co-expressing cells and insulin-producing cells lacking hepatic protein after transdifferentiation (Figure 3.12). This is the first time such a process has been proposed in transdifferentiation studies. As a result of these observations, it is believed that a gap, the demonstration of relationship between the original cell type and the converted cell type, has been filled. This confirmed a genuine transdifferentiation of liver cells to pancreatic cells by introduction of a single "master-switch gene". It is true that the proportion of transfected cells that become differentiated pancreatic cells is very small. This observation suggests that the HepG2 cells may not be homogeneous and therefore have some intrinsic variability of competence, the basis of which is not currently understood.

Results from the current study also demonstrated that transgene expression (as determined by the detection of Vp16 or Xlhbox8) is only transiently required for

transdifferentiation of hepatocytes to pancreatic cells. By the end of the culture period a proportion of pancreatic cells have lost transgene expression and expressed a pancreatic phenotype. A similar effect was also found in the *in vivo* study of Ber et al (Ber et al., 2003). From a clinical point of view, a transient action of the transgene would be beneficial because it would remove the need for transgene integration which might create problems of insertional mutagenesis.

It is not known why the Vp16 domain changes the biological activity of Pdx-1 so profoundly. In this work it is shown that there is no significant effect on the degree of nuclear localisation e.g. by comparison of the proportion of nuclear mPdx1 and mPdx1Vp16 while transfection into HepG2 cells. One factor is probably the fact that Vp16 is a very active transcriptional activation domain so that the change of capacity for transdifferentiation is occurred on the transcriptional level instead of on the activity of nuclear entry of the proteins. Another is that it may be able to act in the absence of the various partner proteins that are used by Pdx-1 in the context of the normal pancreatic cell types (Andersen et al., 1999; Glick et al., 2000; Goudet et al., 1999; Liu et al., 2001).

In the cell-lineage tracing experiment, it was shown that about 50% of insulin-expressing cells are transgene positive, based on the detection of the expression of the DsRed reporter protein (Figure 3.19C). A question that arises is: why don't all of the insulin-producing cells express DsRed? We considered the following reasons might provide an explanation. First, it is possible that insulin-positive cells lose DsRed by the 5^{th} day after transfection. This possibility is based on the observation of a small reduction of DsRed expression in CMV-DsRed transfected cells, as shown in Figure 3.17A. Second, an accumulation of a high level of DsRed proteins in the transfected cells may be inhibitory for transdifferentiation. It is possible for the cells to reduce the amount of DsRed protein either via a mechanism

that involved removal of the protein or active homologous recombination between the two CMV promoters to remove the CMV-DsRed portion from the plasmid. Thirdly, certain "transdifferentiation promoting factors" might be transmitted between the cells and play a role to induce adjacent cells to become pancreatic-like cells. An unknown soluble factor could be secreted from insulin-positive cells (DsRed positive) into medium and consequently taken-up by the other cells (DsRed negative) to induce the endogenous pancreatic programme, for example, expressing insulin protein. Five-day inoculation of the parental HepG2 cells in the culture medium collected from TTR-Xlhbox8Vp16 transfected HepG2 cells was carried out to test this hypothesis. However, no insulin-producing cells, were detected. Lastly, a recent investigation suggested that insulin uptake from the culture medium might be the reason contributing to the detection of insulin-expressing cells derived from other cell types, e.g. embryonic stem cells(Rajagopal et al., 2003). This is unlikely in our case because of the *insulin* mRNA increase (Fig.3.10) and the demonstration of C-peptide in the cells (chapter 4).

Although the results in the present study are encouraging, the transdifferentiation efficiency in the HepG2 cell model needs to be improved. First, use of the high glucose medium might be helpful. Second, establishing a cell line which stably expresses Pdx1Vp16 gene or using a more efficient gene delivery method such as adenovirus infection might serve to ameliorate the low efficiency of transient transfection. Third, the introduction of several pancreatic transcription factors in combination, at appropriate times and dosage manner, to mimic normal pancreas development might increase the percentage of transdifferentiated cells (Soria, 2001; Wang et al., 2001). Fourth, epigenetic manipulation may promote loss of the chromatin structure and thereby enhance the potency of transcription factor for cell reprogramming (Pomerantz and Blau, 2004). For example, the treatment with the

DNA methylation inhibitors 5-aza-cytidine and 5-aza-deoxycytidine might lead to de-condensation of chromatin structure and therefore perhaps more favourable conditions for genetic modification (Goffin and Eisenhauer, 2002; Haaf, 1995).

In summary, the current study shows that in HepG2 cells can be converted into pancreatic-like cells, and we have observed a dynamic process of transdifferentiation by the detection of the changes of hepatic and pancreatic proteins during transdifferentiation.

Chapter 4. Functional properties of transdifferentiated pancreatic-like cells

Overview

The experiments described in Chapter 3 demonstrated that the hepatoma cell line HepG2 can be reprogrammed to pancreatic cells by introduction of a master regulatory gene, Xlhbox8Vp16. Although the transdifferentiated cells displayed some of the characteristics of pancreatic cells (e.g. expression of β-cell markers), the question still remains to what extent the transdifferentiated show the functional properties of β-cells? In order to answer this question, a number of assays were performed.

4.A Induction of insulin processing machinery

4.A.1 Molecules involving insulin biosynthesis

All four pancreatic hormones (insulin, glucagon, somatostatin and pancreatic polypeptide) are initially synthesised as larger prohormones and can be catalytically converted by prohormone convertases (PCs) into smaller, biologically active hormones(Tomita, 2002). For example, proinsulin and proglucagon are the precursors of insulin and glucagon proteins and are post-translationally cleaved into insulin and glucagon by PC1/3 and PC2 (Blache et al., 1994; Tomita, 2002). Proinsulin is preferentially cleaved by PC1/3 at the C-terminal side bond between the C-peptide and B-chain junction whereas PC2 exclusively digests those between the C-peptide and A-chain junction(Smeekens et al., 1992). A reduced activity of PCs is positively correlated to the tumourogenic phenotype of pancreatic endocrine derived tumour cells including insulinomas, glucagonomas and pancreatic polypeptideomas (Tomita,

2001). Mutations in the PC1/3 gene leads to reduced insulin production resulting in disrupted glucose homeostasis in obese females (Jackson et al., 1997). These results indicate that PCs are functionally important for pancreatic endocrine cell types. In particular, PC1/3 is required for the conversion of insulin precursor into functional insulin protein in the insulin producing β-cells.

During the conversion of proinsulin to insulin, an intermediate product, C-peptide, is produced (see section 1.C.1.3). The C-peptide is stored in secretory granules and is released with insulin into circulation system. The physiological role of C-peptide was originally considered as a linker between the A- and B-chain of the insulin protein. In addition, the gene sequence of C-peptide exhibits considerable variation between species in contrast with the highly-conserved mature insulin peptides (Wahren, 2004). From an evolutionary point of view, it might support the idea that C-peptide is not required for normal biological functions. However, recent investigations suggested a role for C-peptide in maintaining glucose homeostasis(Marques et al., 2004). For instance, C-peptide can activate the insulin receptor tyrosine kinase pathway(Grunberger et al., 2001), stimulate glycogen synthesis in hepatocytes and cause insulin-induced cell growth as well as prevent high-glucose stimulated cell apoptosis in human neuroblastoma SH-SY5Y cells (Li et al., 2003b). These studies provide a basis for administration of C-peptide as a potentially effective therapy for diabetes.

4.A.2 Analysis of insulin-processing proteins in transdifferentiated cells

In order to determine whether the insulin biosynthetic machinery is present in the transdifferentiated cells, the expression of prohormone convertase PC1/3, the

predominant active form of PCs in panceatic β-cells, was detected by real-time RT-PCR in transdifferentiated cells. The parental HepG2 cells exhibited no PC1/3 activity while the expression of PC1/3 was induced on the 3rd day following transfection of Xlhbox8Vp16 and increased until the 5th day (Figure 4.1). The detection of PC1/3 coincides with the appearance of pancreatic endocrine genes in the transdifferentiated cells (Figure 3.10).

C-peptide should be produced in transdifferentiated cells assuming the insulin-processing machinery is intact. The co-expression of C-peptide and insulin was examined in control (TTR-Vp16) and Xlhbox8Vp16-transfected HepG2 cells (Dodson and Steiner, 1998). The antibody used for C-peptide detection (see Chapter 2.B.8) reacts with human and mouse C-peptide (Figure 4.2). In the cells transfected with TTR-Vp16 control plasmid, neither human insulin nor C-peptide were expressed whereas the transdifferentiated β cells (characterised by the expression of insulin protein 5 days after TTR-Xlhbox8Vp16 transfection) co-expressed C-peptide protein along with insulin (Figure 4.3). This result confirmed the presence of the insulin processing machinery in transdifferentiated cells.

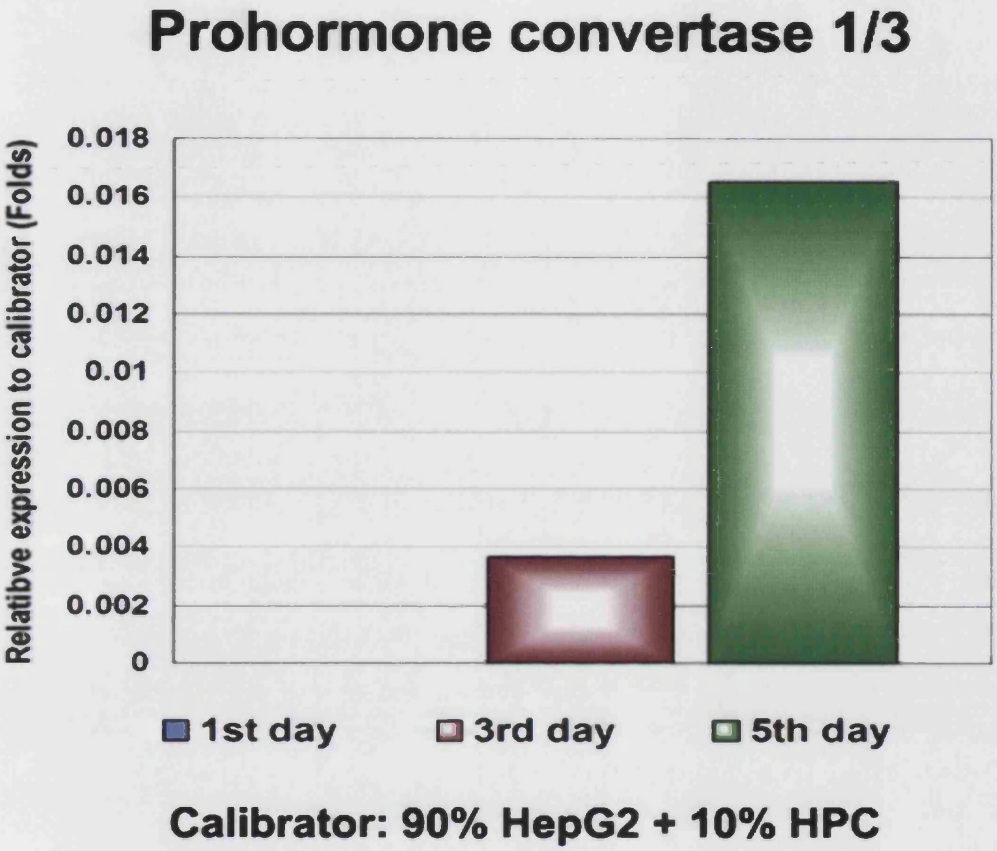


Figure 4.1 Prohormone convertase 1/3 (PC1/3) is detected in HepG2 cells following Xlhbox8Vp16 transfection. Real-time RT-PCR analysis was performed on RNA isolated from transfected cells at the time points indicated. The calibrator used is the cDNA generated from the mixture of 90% HepG2 cells plus 10% human pancreatic cells.

Figure 4.2

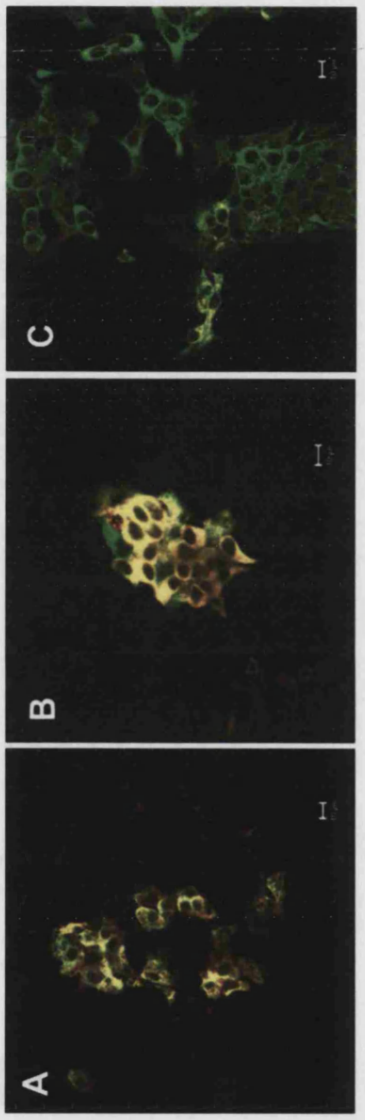


Figure 4.3

Figure 4.2 Immunofluorescence detection of C-peptide and insulin in the pancreatic tissues derived from different species. C-peptide is detected using the anti-human C-peptide antibody on (A) human pancreatic human tissues; (B) 7-day cultured E11.5 dorsal pancreatic buds but not on (C) rat insulinoma cell line, RIN-m5F.

Figure 4.3 C-peptide and insulin colocalisation in transdifferentiated cells. The immunofluorescence staining showed that human C-peptide and insulin are co-expressed in transdifferentiated cells after 5 days of Xlhbox8Vp16 (B) but neither is seen in the control (TTR-Vp16) transfected cells (A).

4.B Responsiveness for physiological stimuli of transdifferentiated cells

A number of physiological stimuli regulate maturation of function of the pancreatic β cells. These stimuli include high glucose, glucagon-like peptide 1 (GLP-1) and betacellulin (Li et al., 2004; Meier and Nauck, 2005). The effects of each of these factors were tested on the Xlhbox8Vp16 transfected cells to further characterise the functional properties of the transdifferentiated β-cells.

4.B.1 Effect of GLP-1

4.B.1.1 Physiological effect of GLP-1 on pancreatic β cells

GLP-1 is encoded within the proglucagon gene and is synthesised in the enteroendocrine intestinal L-cells of the distal ileum and colon by tissue-specific posttranslational processing (Figure 4.4) (Baggio and Drucker, 2004; Drucker, 2001). In response to nutrient intake, GLP-1 secretion is rapidly increased via the neuronal stimulation of the vagus nerve (Baggio and Drucker, 2004). Native GLP-1 is a polypeptide composed of 37 amino acids and the half-life of circulating biologically active GLP-1 is less than 2 minutes because of the rapid degradation by a ubiquitously-distributed membrane-associated peptidase, dipepidyl peptidase IV (DPP-IV) (Kieffer et al., 1995). The physiological effect of GLP-1 is of immense interest due to its effect on glucose homeostasis, i.e. decreasing insulin resistance in peripheral tissues such as muscle and increasing the insulin secretion from β cells (D'Alessio et al., 2005; Meier et al., 2002). GLP-1 exerts a wide range of extrapancreatic effects. For example, GLP-1 delays gastric empting, slows small intestine motility (Giralt and Vergara, 1999) and also increases glycogen synthesis in

the liver, adipose tissues and skeletal muscles (Valverde et al., 1994). In addition, GLP-1 exhibits a profound influence in controlling appetite and weight by neuronal regulation, a notion based on the expression of GLP-1 receptor within the anterior hypothalamic nuclei involved in appetite regulation in brain (Alvarez et al., 1996; Holst, 2000). Acute administration of GLP-1 leads to satiety and decreases food intake whereas the introduction of a truncated form of GLP-1, the antagonist exendin 9-39, neutralises the effect of the GLP-1 and promotes weight gain (Flint et al., 1998; Meeran et al., 1999). Reduced food intake and appetite has also been observed in human type 2 diabetic patients after short-term treatment with GLP-1 or its long-term agonist, exendin 4 (Toft-Nielsen et al., 1999).

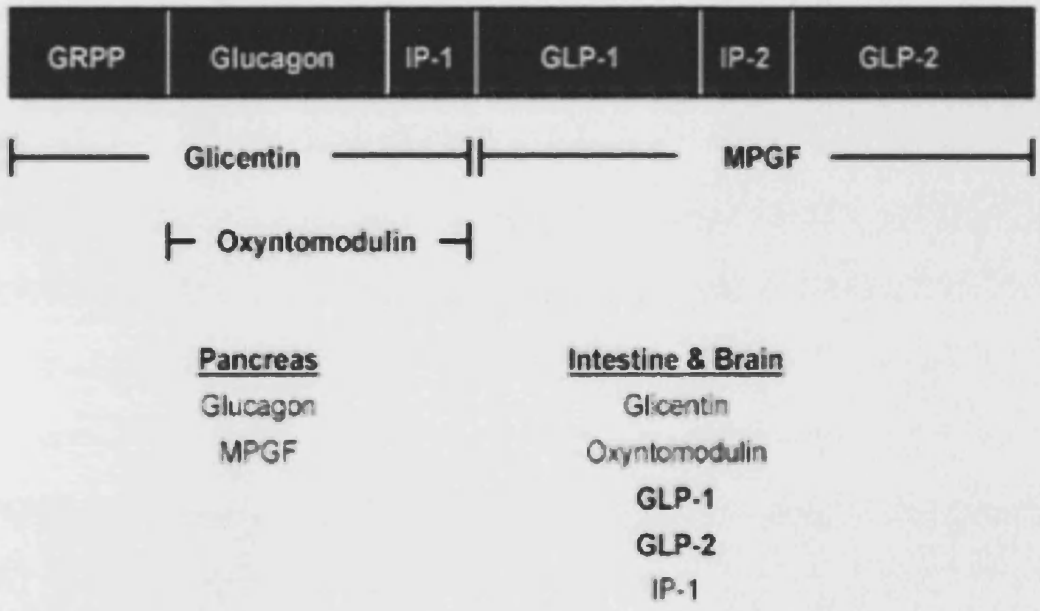


Figure 4.4 Diagrammatic representation of mammalian proglucagon structure. The tissue-specific post-translational processing of the proglucagon-derived peptides. GRPP, glicentin-related polypeptide; IP-1, intervening peptide-1; IP-2: intervening peptide-2; MPGF, major proglucagon fragment; GLP-1 and GLP-2, glucagon-like peptide-1 and -2 is also shown. Reproduced from Baggio & Drucker, 2004.

The actions of GLP-1 in the pancreas to govern glucose homeostasis have generated much interest and the mechanisms underlying blood glucose regulation by GLP-1 are now much understood. The effect of GLP-1 is mediated via the activation of the G-protein coupled receptor that is highly expressed in pancreatic β cells (Thorens, 1992). A sequence of intracellular signalling events is activated following GLP-1 receptor binding, resulting in the increase of insulin biosynthesis, the enhancement of proliferation and differentiation as well as the inhibition of apoptosis of pancreatic β cells (Gromada et al., 2004; List and Habener, 2004). Adenylate cyclase is activated when GLP-1 binds the receptor thereby inducing cyclic 5'-adenosine monophosphate (cAMP) production. In turn, the accumulation of cAMP activates the protein kinase A (PKA) signalling pathway and sequentially closes K_{ATP} channels. This blockage of efflux of potassium ions depolarises the cell membrane and directs a shift of intracellular Ca^{2+} concentration through the opening of voltage-gated calcium channel (VGCC). The resulting prolonged action potential of the membrane positively regulates the intracellular ATP/ADP ratio. At the same time, the voltage-gated K^{+}-channels (Kv) are also inhibited by GLP-1 hence strengthening the action potential. The active PKA signalling also induces Ca^{2+} release from the ER mediated by inositol-1,4,5-triphosphate (IP_3), a by-product of the breakdown of phosphatidylinositol-4,5-biphosphate (PIP_2) in the cell membrane. The pumping of Ca^{2+} ions into cells triggers insulin granule mobilisation and subsequent insulin exocytosis. Furthermore, it has been suggested that GLP-1 inhibits cell apoptosis via a PKA- and phosphatidylinositol 3-kinase (PI3K)- pathway in mouse insulinoma MIN6 cells(Hui et al., 2003). Additionally, GLP-1 can also improve insulin resistance in diabetic subjects(Sandhu et al., 1999).

GLP-1 is capable of converting other cell types into pancreatic endocrine cells, particularly β cells. In other words, GLP-1 also has the ability to promote the

differentiation of endocrine cells. The expression of insulin and Pdx-1 as well as glucagon or pancreatic polypeptide were induced or up-regulated after the treatment of GLP-1 or related agonist exendin 4 in some cell lines including the rat pancreatic tumour derived AR42J cells, the rat ductal cell line ARIP and the human pancreatic adenocarcinoma Capan-1 cells (Hui et al., 2001; Zhou et al., 2002; Zhou et al., 1999). It is also noteworthy that Pdx-1 negative cells, such as human pancreatic ductal cells Panc-1, are able to become insulin-producing cells after the treatment with GLP-1 in combination with the introduction of Pdx-1(Hui et al., 2001). These results suggested that the effect of GLP-1 on transforming non-endocrine cells into endocrine cells is mediated via a Pdx-1 dependent mechanism (Li et al., 2005). This hypothesis was further confirmed by a recent publication describing the conversion of intestinal cells (Pdx-1 positive) into insulin-producing cells by the administration of GLP-1 (Suzuki et al., 2003).

4.B.1.2 GLP-1 receptor is induced in transdifferentiated cells

From the description above, it is clear that normal pancreatic cells are responsive to GLP-1. Assays to determine the sensitivity of transdifferentiated cells to GLP-1 were carried out. Since the action of GLP-1 is mediated through binding with the GLP-1 receptor, transdifferentiated cells were first examined for GLP-1 receptor expression using real-time RT-PCR. Low-level expression of GLP-1 receptor was detected in parental HepG2 cells (Figure 4.5). Five days after transfection with Xlhbox8Vp16 the GLP-1 receptor mRNA was upregulated by ~5.5 fold in comparison with the calibrator (Figure 4.6).

The expression of insulin mRNA in transdifferentiated cells was then tested using the following protocol. The HepG2 cells were transfected with either TTR-Vp16 or TTR-Xlhbox8Vp16 DNA and maintained for 5 days. The transdifferentiated cells were

then treated with 10nM GLP-1 for 12, 36 and 60 hours. This concentration of GLP-1 was previously shown to induce optimal insulin secretion activity in converted Panc-1 cells after treatment of GLP-1 (Hui et al., 2001). The RNA was collected and real-time RT-PCR for the insulin gene was performed. It was shown that the level of insulin mRNA is stimulated about 6x by GLP-1 in the transdifferentiated cells (Figure 4.7).

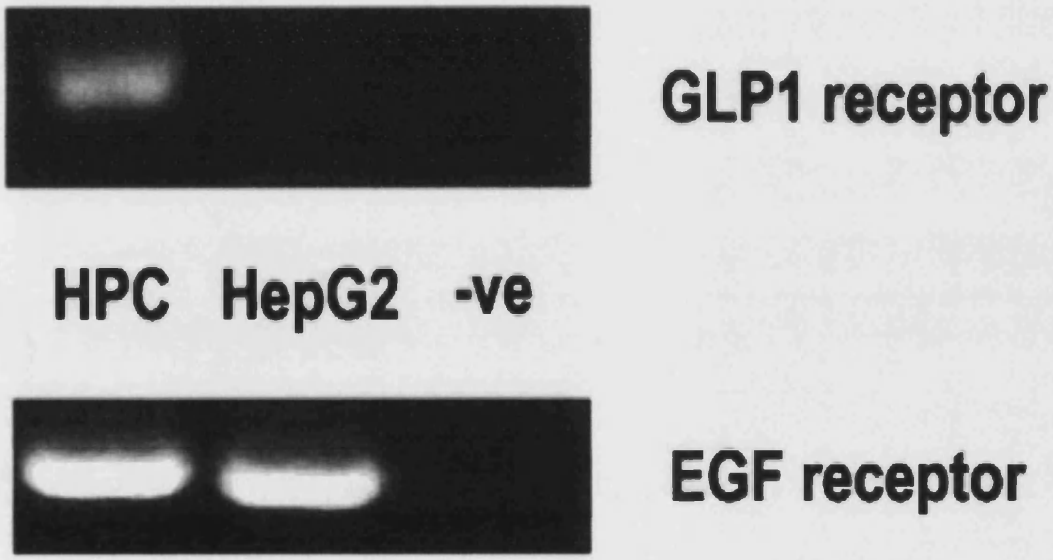


Figure 4.5 Expression of Glucagon-like peptide 1 receptor (GLP1R) and Epidermal growth factor receptor (EGFR). GLP1R is expressed abundantly in human pancreatic cells (HPC) but is only weakly detected in HepG2 cells. EGFR is massively expressed in both HPC and HepG2 cells. –ve: no template control.

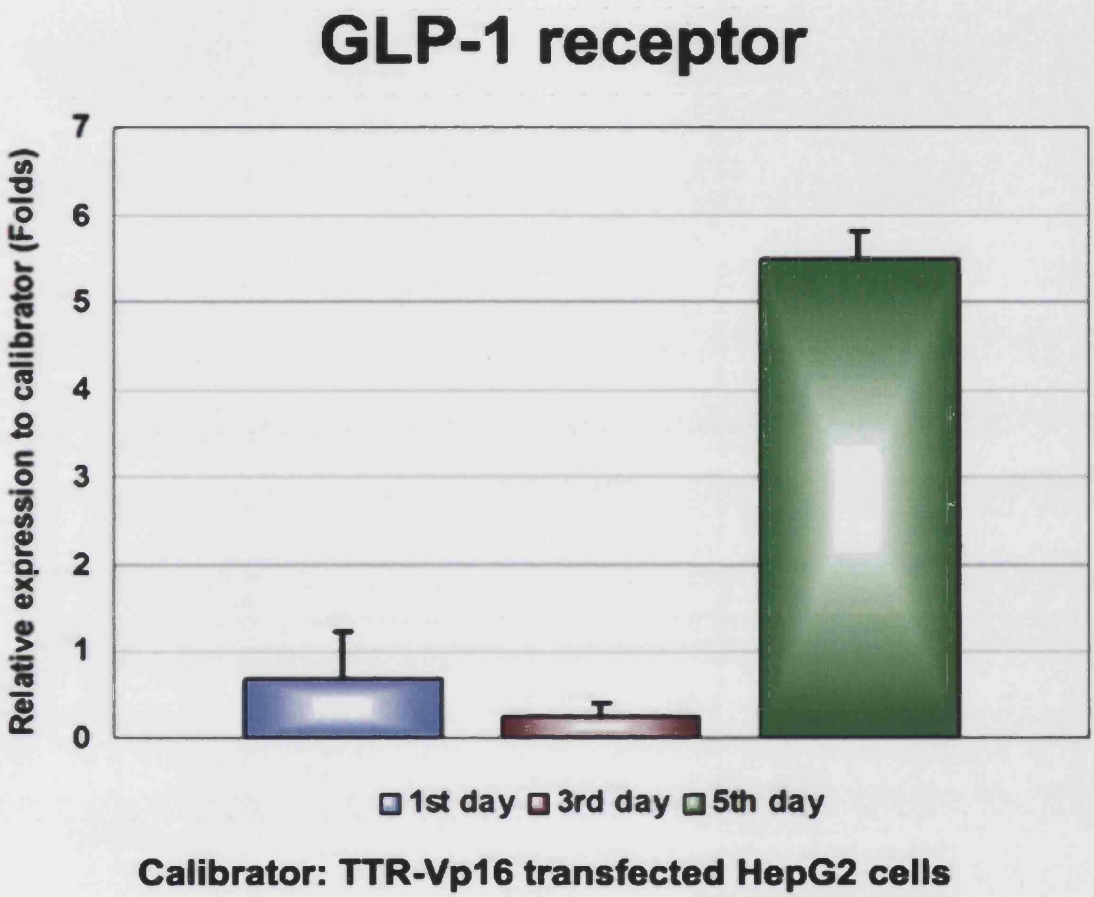


Figure 4.6 GLP1R is upregulated during transdifferentiation. Real-time analysis examined GLP1R mRNA expression. A sharp increase is detected between the 3rd and 5th day after transfection of Xlhbox8Vp16. The calibrator used is TTR-Vp16 transfected HepG2 cells and the results are presented as mean + S.D.

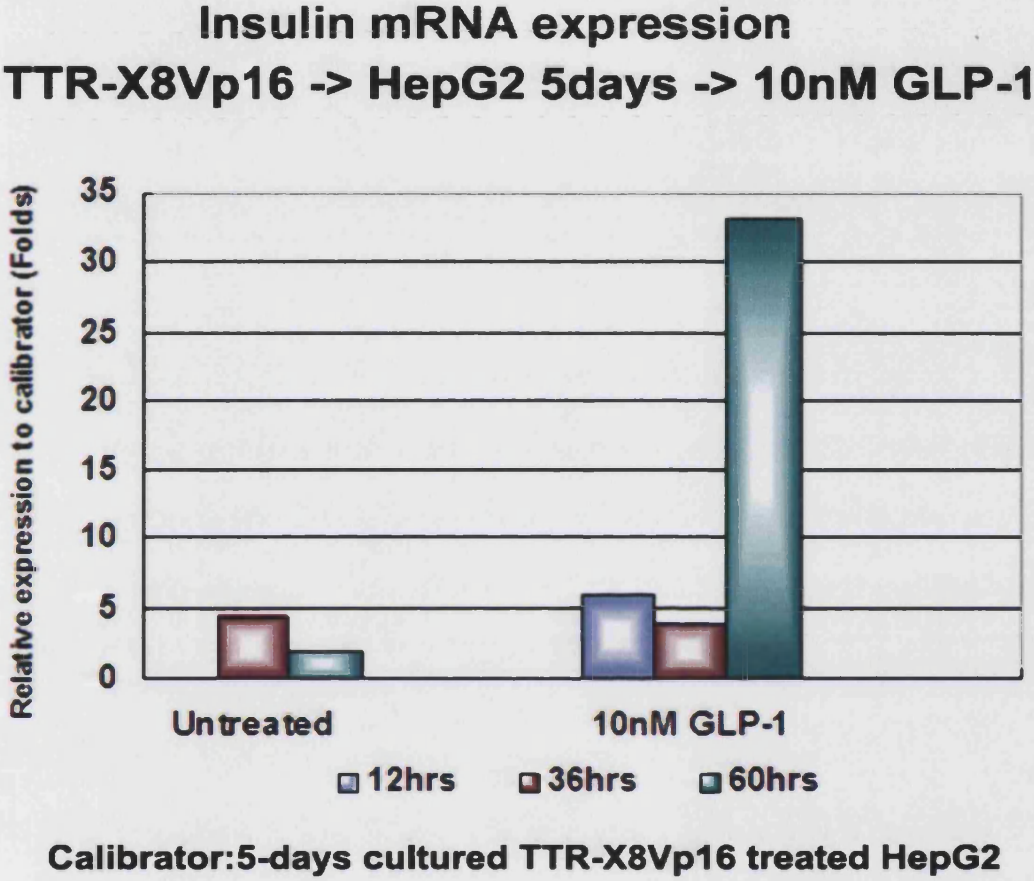


Figure 4.7 Time-dependent upregulation of insulin mRNA in transdifferentiated cells following GLP-1 treatment. The HepG2 cells were transfected with the TTR-Xlhbox8vp16 construct and maintained in culture for 5 days. 10nM GLP-1 is then added for 12, 36 and 60 hours. Insulin mRNA was examined by real-time RT-PCR. In comparison to the untreated cells, GLP-1 treated cells express higher levels of insulin mRNA. The calibrator used is 5-day TTR-Xlhbox8Vp16 transfected HepG2 cells.

4.B.2 Effect of betacellulin

4.B.2.1 Physiological effect of betacellulin on pancreatic β cells

Betacellulin (BTC) was first characterised in the conditioned medium of a cell line derived from a mouse pancreatic β cell tumour(Shing et al., 1993). It belongs to a member of the epidermal growth factor (EGF) family and it is highly expressed in the pancreas, liver, kidney and small intestine (Dunbar and Goddard, 2000; Seno et al., 1996). Structurally, BTC possesses six cysteine consensus motifs that form three intramolecular disulfide bonds (Dunbar and Goddard, 2000). BTC binds to the EGF receptor (EGFR) including the homodimers of EGFR / erbB1 and erbB4 or heterodimers of various erbB receptors to mediate intracellular signalling (Graber et al., 1999; Huotari et al., 2002; Ishiyama et al., 1998; Sundaresan et al., 1998).

Several studies have shown that BTC stimulates the growth and differentiation of pancreatic cells both *in vitro* and *in vivo* and is therefore regarded as a mitogen for pancreatic β cells (Huotari et al., 1998). The most compelling evidence for this comes from studies on cell lines. Conversion of pancreatic exocrine-derived AR42J cells to insulin producing cells can be induced by culture with BTC and activin A, a member of the transforming growth factor β (TGF-β) family (Mashima et al., 1996). Both the pancreatic α cell line, α-TC1.6 and the intestinal epithelial cell line IEC-6 can be transformed into insulin-producing cells by BTC treatment in the presence of Pdx-1 (Watada et al., 1996b; Yoshida et al., 2002). More recently, the insulinotropic effect of BTC on pancreatic islet cells was demonstrated *in vitro.* For instance, Demeterco et al. showed that BTC caused proliferation in human foetal pancreatic tissues as previously observed in AR42J cells(Demeterco et al., 2000). BTC activated the

regeneration and neogenesis of β cells and sequentially improved hyperglycaemia in alloxan or streptozotocin diabetic mice and in 90% pancreatectomised rats (Li et al., 2001; Li et al., 2003a; Yamamoto et al., 2000). These data confirm that pancreatic β cells are sensitive to BTC.

4.B.2.2 Betacellulin up-regulates insulin mRNA expression

The responsiveness of transdifferentiated HepG2 cells to BTC treatment was examined using RT-PCR for *insulin* mRNA. In order for BTC to elicit a response, it is necessary that the EGFR is expressed and this was shown to be the case in HepG2 cells (Figure 4.5). To determine the effect of BTC on transdifferentiated cells HepG2 cells were transfected with Xlhbox8Vp16 and then treated for 60 hours with 10 ng/ml BTC. There was a dramatic increase (about 25-fold) in expression of insulin mRNA compared to calibrators (Figure 4.8). The effect of BTC on transdifferentiated cells is much greater than that of GLP-1. This observation implies that transdifferentiated cells behave in the same manner as normal pancreatic cells and are actively responsive to BTC.

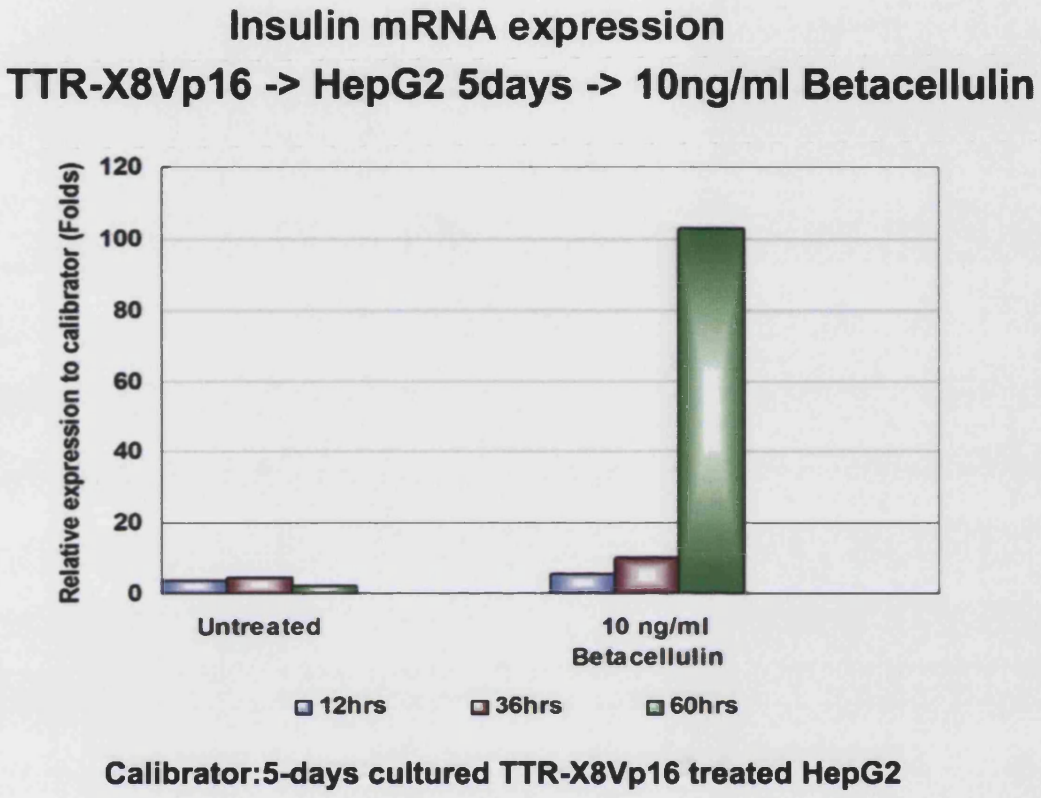


Figure 4.8 Time-dependent upregulation of insulin mRNA in transdifferentiated cells after betacellulin treatment. The HepG2 cells were transfected with the TTR-Xlhbox8vp16 construct and maintained in culture for 5 days. 10ng/ml betacellulin was then added for 12, 36, and 60 hours. Insulin mRNA was detected by real-time RT-PCR. In comparison to the untreated cells, GLP-1 treated cells express higher levels of insulin mRNA. The calibrator used is 5-day TTR-Xlhbox8Vp16 transfected HepG2 cells.

4.B.3 Glucose-stimulated insulin release (GSIR) in transdifferentiated cells

In terms of β cell function, the most important characteristic property is the ability to secrete insulin in response to an elevation of external glucose (discussed in section 1.C.1.2). In order to determine whether the transdifferentiated cells can perform this essential function, an ultrasensitive insulin ELISA kit was used. Although the proportion of insulin-positive cells as a fraction of the total cells in the transfected HepG2 cell system was low, it was possible to demonstrate glucose-stimulated insulin release. The insulin was detected in the medium of 5-day Xlhbox8Vp16-transfected cells after a challenge with 25 mM glucose. The results showed that there is a reproducible and significant increase of insulin secretion. No increase in GSIR was seen for transfected cells exposed to low glucose and for control (TTR-Vp16) transfectants, either exposed to low or high glucose (Figure 4.9).

4.C Discussion

The data presented in this chapter shows that the transdifferentiated cells display several functions of pancreatic β cells. These include expression of PC1/3 and C-peptide, indicative of processing of proinsulin; response to physiological stimuli such as GLP-1 and betacellulin; and release of insulin in response to glucose. These results indicate that transdifferentiated cells can respond in a similar fashion to normal pancreatic β cells. One question that remains unanswered is whether transdifferentiated cells show biphasic insulin secretion. However, due to the limited number of transdifferentiated cells, this was not possible to perform. Additionally, the presence of insulin in the serum of the culture media makes it more difficult to measure and interpret whether biphasic insulin secretion exists in transdifferentiated

cells.

Transdifferentiated cells might provide a treatment for diabetes (Meivar-Levy and Ferber, 2003). However, in order for the therapeutic potential of transdifferentiated cells to be useful, a number of criteria must be fulfilled (Weir, 2004). For example, the surrogate β cells must exhibit all specific functional properties that β cells normally exhibit. In addition, transdifferentiated β cells should also have the repopulation potential to satisfy the demands of transplantation for curing diabetic patients. One of the practicable methods to achieve this future goal is to establish a stable cell line expressing the master switch gene, i.e. Xlhbox8Vp16. However, there was not time to do this in the present work. Moreover, although HepG2 cells have prolonged proliferation capacity, they also show carcinogenic properties. The introduction of an immortalising gene, e.g. telomerase catalytic subunit hTERT (Poole et al., 2001), into normal cells to expand the life span, or using the cells of foetal origin might be a way to develop a long-term non-carcinogenic stable β-cell-like cell line. Recently, it has been shown that foetal liver progenitor cell lines expressing Pdx1 exhibit pancreatic phenotypes and can rescue diabetic hyperglycaemia (Zalzman et al., 2005; Zalzman et al., 2003).

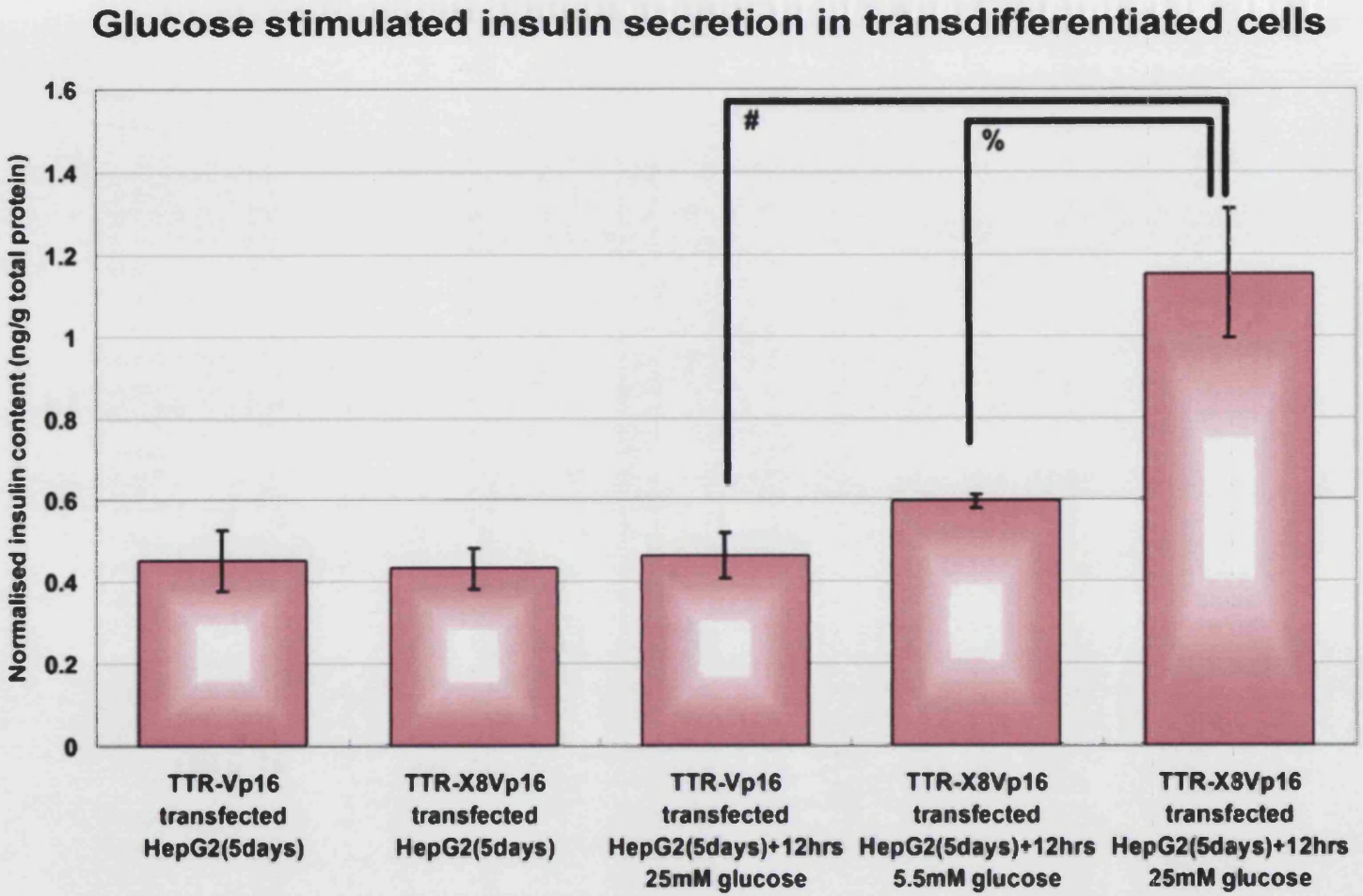

Glucose stimulated insulin secretion in transdifferentiated cells
Normalised insulin content (ng/g total protein)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
#
%
TTR-Vp16 transfected HepG2(5days)
TTR-X8Vp16 transfected HepG2(5days)
TTR-Vp16 transfected HepG2(5days)+12hrs 25mM glucose
TTR-X8Vp16 transfected HepG2(5days)+12hrs 5.5mM glucose
TTR-X8Vp16 transfected HepG2(5days)+12hrs 25mM glucose
P-value #: 0.000298696<0.005; %:0.001391332<0.005

Figure 4.9 Transdifferentiated β-cells secrete insulin in response to glucose. Control cells (TTR-Vp16 transfected cells and cultured for 5 days) or transdifferentiated cells (cells transfected with TTR-Xlhbox8Vp16 and cultured for 5 days) were treated with physiological (5.5mM) or higher concentrations (25mM) of glucose for 12 hours. The medium is collected and the secreted insulin is detected using an insulin ELISA kit. Following treatment with high concentrations of glucose, transdifferentiated cells significantly release more insulin compared with the control cells. The data are represented as mean ± S.D. from 3 independent experiments.

Chapter 5. Establishment of a culture system for embryonic liver

Overview

The results presented in Chapters 3 and 4 demonstrated that it is possible to induce the transdifferentiation of HepG2 cells to pancreatic cell types by overexpression of the Xlhbox8Vp16 transgene. More specifically, it was shown that the pancreatic cells (i) express markers characteristic of β cells, (ii) are functional, and (iii) that the transgene need not remain active in the long term (this is known as the hit and run hypothesis). However, HepG2 cells do not exhibit an entirely normal hepatic phenotype. For instance, HepG2 cells lack some of the liver enriched transcription factors (e.g. C/EBPα) and some hepatic proteins (e.g. multidrug resistant protein (MRP)) (Figure 3.1). Therefore, the question arises whether transdifferentiation can be induced in primary cultures of liver cells using the Xlhbox8Vp16 reagent?

To address this problem, we decided to establish a reliable and well-defined *in vitro* culture system for primary liver cells. In this chapter, the results from the first model based on culture of embryonic liver are presented. It was chosen to examine embryonic liver because the tissue may exhibit greater plasticity compared to the adult. In addition, development of an *in vitro* culture system for embryonic liver may provide a useful tool to study normal liver development.

5.A Isolation of embryonic liver cells

The embryonic liver originates from the epithelium of the anterior endoderm (see section 1.A.2). During development, the liver is surrounded by a mass of mesenchymal cells and signals such as BMPs and FGFs, are secreted from the mesenchymal cells. These signals are essential for the initiation, expansion and maturation of hepatocytes. Based on the importance of mesenchymal cells around the fetal liver as well as previous experience in culturing of embryonic pancreatic tissues

in the laboratory (Percival and Slack, 1999), direct isolation of whole hepatic epithelial region from E11.5 embryo was carried out (shown in Figure 2.1). The project was designed as shown in Figure 5.1.

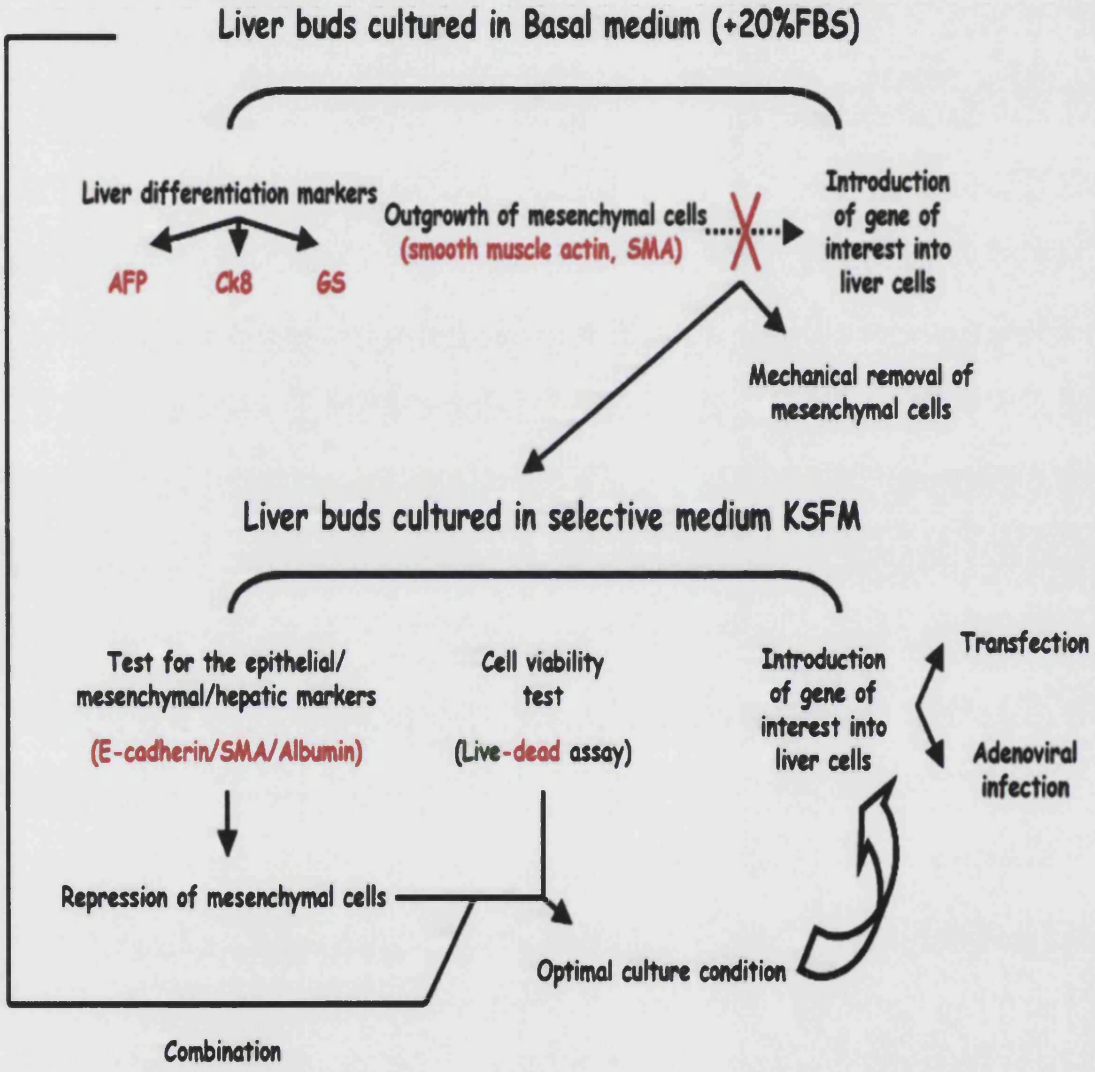


Figure 5.1 Procedure for the *in vitro* culture of embryonic liver.

5.A.1 Liver cells cultured in BME with 20% FBS medium

The initial experiments on embryonic liver culture were performed in basal medium containing Earle's salt and 20 % FBS. Following isolation, the cells attached and began to spread out on the fibronectin-coated coverslips by 24 hrs (Figure 5.2). We examined the expression of three hepatic markers for either embryonic or mature liver cells to detect the differentiation status of the cells during different periods of culture. The markers used were α-fetoprotein (AFP, to detect immature hepatoblasts) (Abelev and Eraiser, 1999), cytokeratin 8 (CK8) (to detect epithelial cell types) and glutamine synthetase (GS, to detect mature hepatocytes) (Bennett et al., 1987; Ku et al., 2001; Omary et al., 2002; Smith and Campbell, 1988). Results indicated that AFP expressing cells gradually decreased after 7 days of culture compared to those in culture for only 2 or 5 days. In contrast, the number of GS-expressing cells increased when the cells were cultured for longer periods (the GS protein accumulation begins normally in mouse hepatocytes at about E15 (Notenboom et al., 1997)). CK8 was detected on the 3rd day of culture and expression was maintained throughout the remaining culture period up to 7 days (Figure 5.3). This evidence suggests that the BME culture system may be a useful tool in the study of the liver development *in vitro*.

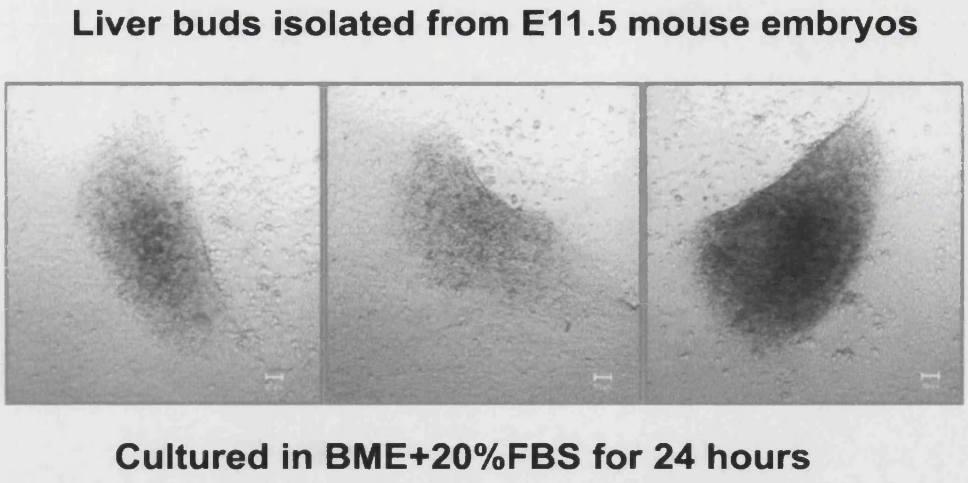


Figure 5.2 A transmitted light image of mouse embryonic liver tissues cultured for 24hrs in basal medium with Earle's salts in the presence of 20% FBS. The liver tissues are isolated from E11.5 mouse embryos and cultured on a fibronectin-coated coverslip. A representative image is shown. Scale bar = 50 μm

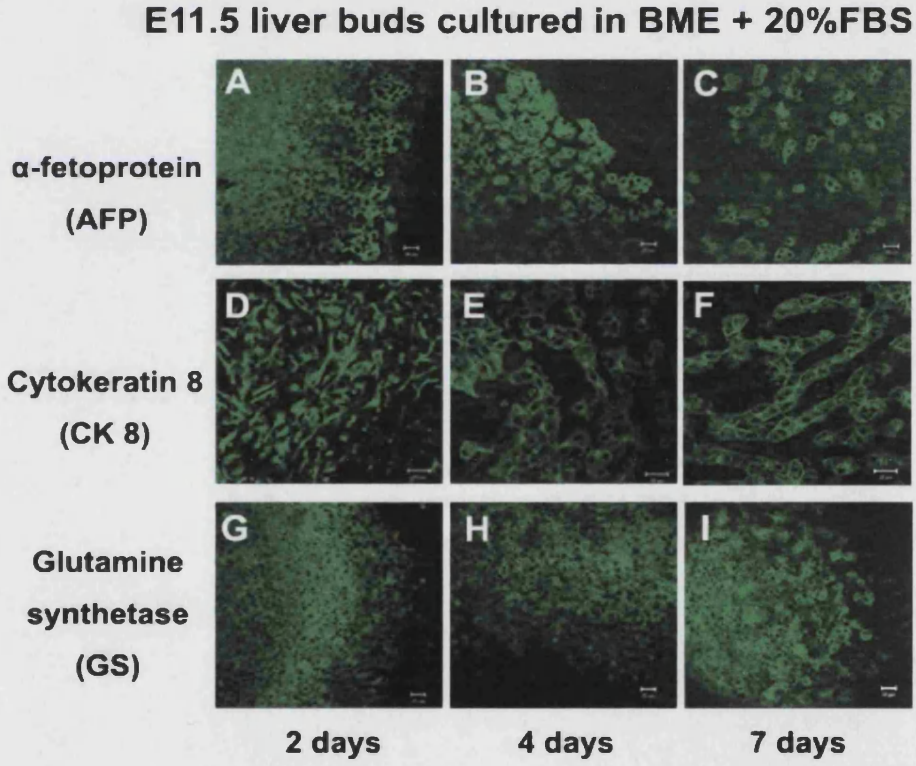


Figure 5.3 Time course analysis of liver markers in embryonic liver tissues. Liver tissues were isolated from E11.5 mouse embryos and cultured in BME (with 20% FBS) for 2, 4 and 7 days on fibronectin-coated coverslips and immunostained for α-fetoprotein (A-C), cytokeratin 8 (D-F) and glutamine synthetase (G-I). Scale bar = 20 μm

5.A.2 Transfection of hepatic epithelial cells is prevented by mesenchymal cells

To induce transdifferentiation of embryonic liver cells to pancreatic-like cells efficient delivery of the XlhboxVp16 DNA into hepatic epithelial cells is required. Among the various gene delivery methods including transfection, electroporation or viral infection, it has been considered that adenoviral infection is the most efficient (Barnett et al., 2002). Adenovirus has a typical morphology with an icosahedral capsid consisting of 3 major proteins including hexon, penton base and a knobbed fibre. The virus genome is linear, double- strained DNA ending with a terminal protein attached to the inverted terminal repeats (Figure 5.4)(Russell, 2000). Adenoviral infection is mediated through the high-affinity conjugation of the fibre knob with the widely distributed cellular receptor called the coxsackievirus and adenovirus receptor (CAR)(Bergelson et al., 1997) and the binding between the arginine-glycine-aspartic acid (RGD) residue of penton base and integrins αvβ3 or αvβ5 (Wickham et al., 1993).

The titre of the adenoviral vector used to infect embryonic liver cells was first tested in order to calculate the optimal infection conditions. As shown in Figure 5.5, using adenovirus encoding CMV-GFP DNA sequence, 10^8 virus particles were sufficient to obtain a good infection based on GFP expression. However, the GFP was mainly present in the overlying mesenchymal cells rather than in the deeper epithelial region. To confirm this observation, and to exclude the possibility that the problem was virus-dependent, the embryonic liver cultures were infected with another adenoviral vector containing CMV-*LacZ* DNA. After infection, it was co-stained for β-galactosidase, the protein product of the LacZ gene, and E-cadherin (the epithelial marker) in the infected cells. If the virus only infected mesenchymal cells, no

β-galactosidase/E-cadherin coexpressing cells should be found. Using the Z-series function on the confocal microscope, it was shown that the hepatic epithelial cells did not receive any adenoviral particles (Figure 5.6). This means that under BME culture conditions, epithelial infection is prevented by the overlying mesenchymal cells.

5.A.3 Mechanical removal of mesenchymal cells from liver culture

To improve the infection efficiency of BME cultured liver tissue, the mesenchyme must be removed from the epithelium so that the virus can reach onto the epithelial cells. The removal of the mesenchyme was carried out after the liver tissue had been in culture for 24 hours. The reason for doing it this way was because immediately after isolation the epithelium and mesenchyme are difficult to distinguish. However, after culture, the mesenchyme forms a layer over the underlying epithelium that in theory can be peeled off. The mesenchyme was removed by mechanical operation using fine forceps. Although mesenchymal and epithelial cells appeared to be successfully separated (Figure 5.7), the infection efficiency was only slightly improved compared to the situation when the mesenchyme and epithelium were not separated (Figure 5.8). This observation suggested that the mechanical removal is not sufficient to completely separate all the mesenchymal cells from the epithelium.

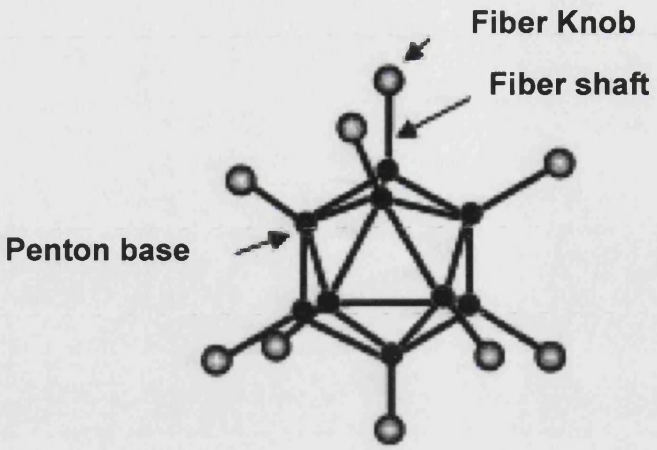


Figure 5.4 Diagrammatic representation of the structure of adenovirus. Reproduced from Barnett et al, 2002.

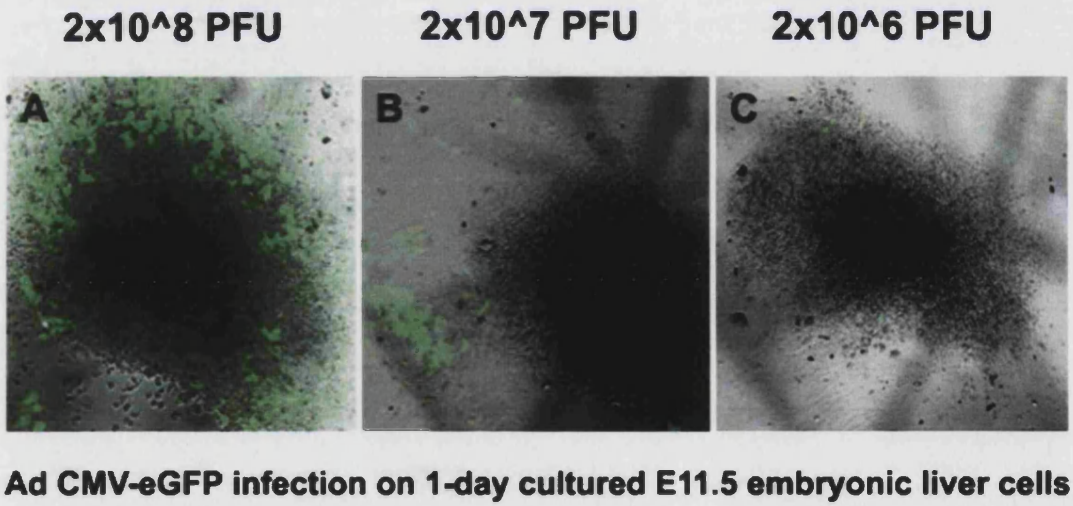


Figure 5.5 Adenoviral infection of 24-hour cultured liver tissues. Liver tissues were isolated from E11.5 mouse embryos and infected with the titres of Ad CMV-eGFP indicated. The images were taken on the following day after infection. PFU, plaque forming units.

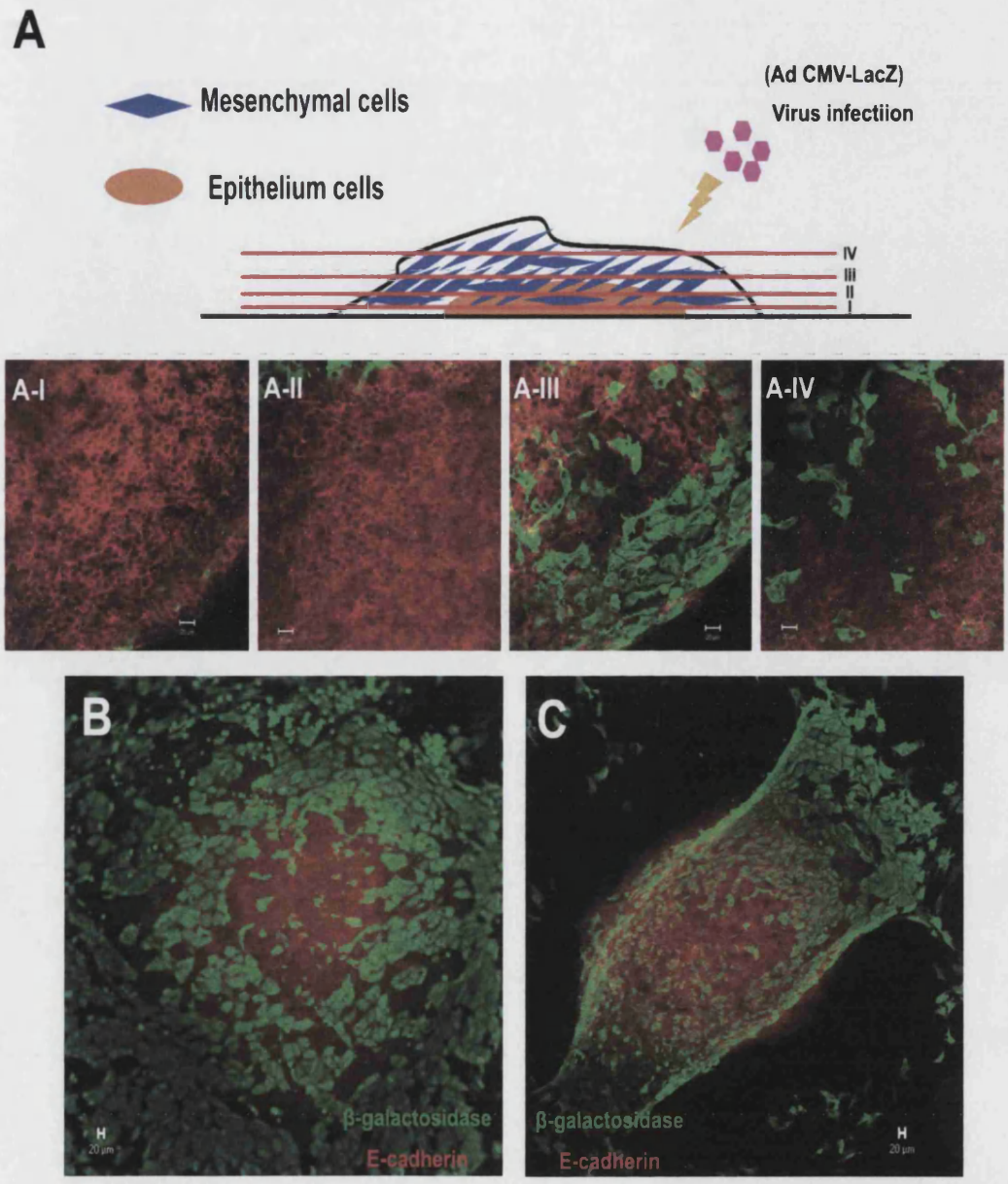


Figure 5.6 Infection of adenovirus CMV-LacZ onto isolated embryonic liver tissues cultured in BME and 20%FBS. (A) Diagrammatic representation of the infection procedure. The embryonic liver tissues were isolated from E11.5 mouse embryos and infected with 10^8 PFU virus particles for 1 hour. The liver tissue was then maintained in fresh BME and 20% FBS for a further 24 hours. A-I to A-IV shows images taken through different layers of the tissue using the Z-series collection facility on the LSM 510 confocal microscope. (B,C) the absence of infected adenoviral vector in the epithelial cells of embryonic liver tissues. The cells are stained with β-galactosidase (green) and E-cadherin (red). Scale bar = 20 μm

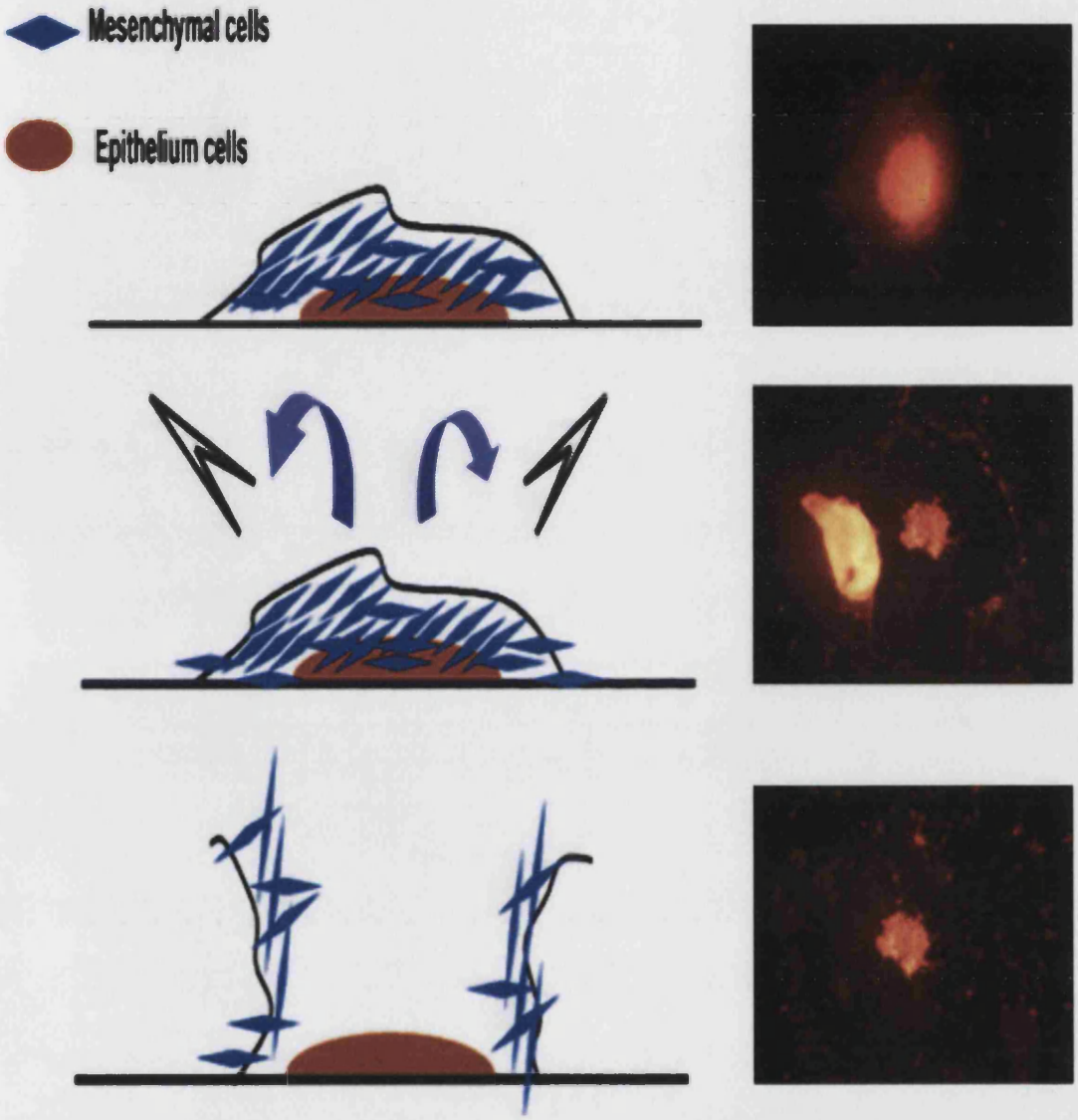


Figure 5.7 Schematic showing the removal of mesenchymal cells from cultured embryonic liver tissues. Isolated liver tissues (from E11.5 mouse embryos) were cultured in BME and 20% FBS for 24 hours after isolation. The mesenchymal cells were removed using sharp forceps to expose the underlying epithelial cells.

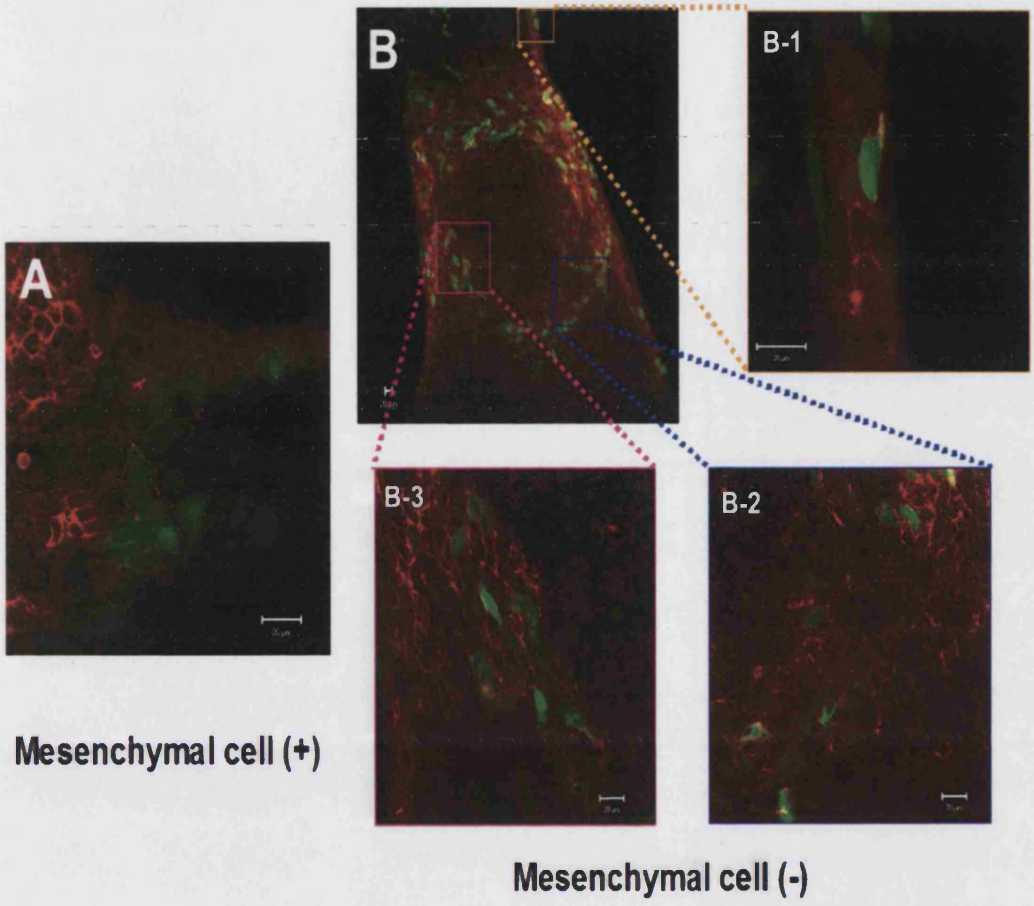


Figure 5.8 Adenoviral infection of cultured embryonic liver tissues either with (A) or without (B) mesenchymal tissue. The cultured embryonic liver cells were isolated from E11.5 mouse embryos and cultured for 24 hours in BME and 20% FBS. On the following day, the mesenchymal cells were mechanically removed (as shown in Figure 5.7) and the epithelium was then infected with 10^8 PFU of Ad CMV-eGFP. E-cadherin (red) and GFP (green) was detected by immunofluorescence staining. Scale bar = 20 μm

5.B Culture of embryonic liver cells in selective medium

5.B.1 Keratinocyte serum free medium (KSFM)

Certain media can selectively favour the growth of specific cell types, such as epithelial cells. Using selective medium might improve the viral infection of a gene product into embryonic hepatic epithelial cells. Several groups had previously used KSFM for culturing a wide range of epithelia, such as oral, oesophageal, corneal, foreskin, bronchial and gingival epithelial cells (Andl et al., 2003; Chen et al., 2002; Duits et al., 2003; Kremer et al., 1999; Lin et al., 2005; Vallette et al., 1998). Therefore, it was decided to test whether KSFM can maintain hepatic epithelial cells and at the same time, repress the growth of mesenchymal cells.

5.B.2 Repression of mesenchymal cells in KSFM medium

There are two kinds of commercially available KSFM that differ in the concentration of Ca^{2+}. One contains no Ca^{2+} (0mM) and the other contains a low concentration Ca^{2+} (0.9mM). The low concentration form is favourable for the growth of epidermal cells (Hennings et al., 1980). The two media were tested to determine their ability to maintain the hepatic epithelial phenotype in isolated embryonic liver. It was examined the expression of albumin, E-cadherin and smooth muscle actin as hepatic, epithelial and mesenchymal cell types, respectively. The results showed that both media actively inhibit mesenchymal cell growth based on lack of smooth muscle actin expression. However, loss of E-cadherin expression in cells cultured in KSFM

without Ca^{2+} confirmed that they do require Ca^{2+} in low amounts to preserve the epithelial phenotype in an *in vitro* culture system (Figure 5.9 & Figure 5.10). It was shown that the growth of the mesenchymal cells in BME medium is much greater than the cells cultured in KSFM medium (Figure 5.11 & Figure 5.12).

Not only does the KSFM inhibit the growth of mesenchymal cells but also inhibits the time-dependent migration of the mesenchymal cells out from the central area (Figure 5.13). Therefore, based on the maintenance of epithelial and hepatic properties and the repression of mesenchymal phenotype, KSFM medium with low Ca^{2+} was used for all subsequent experiments.

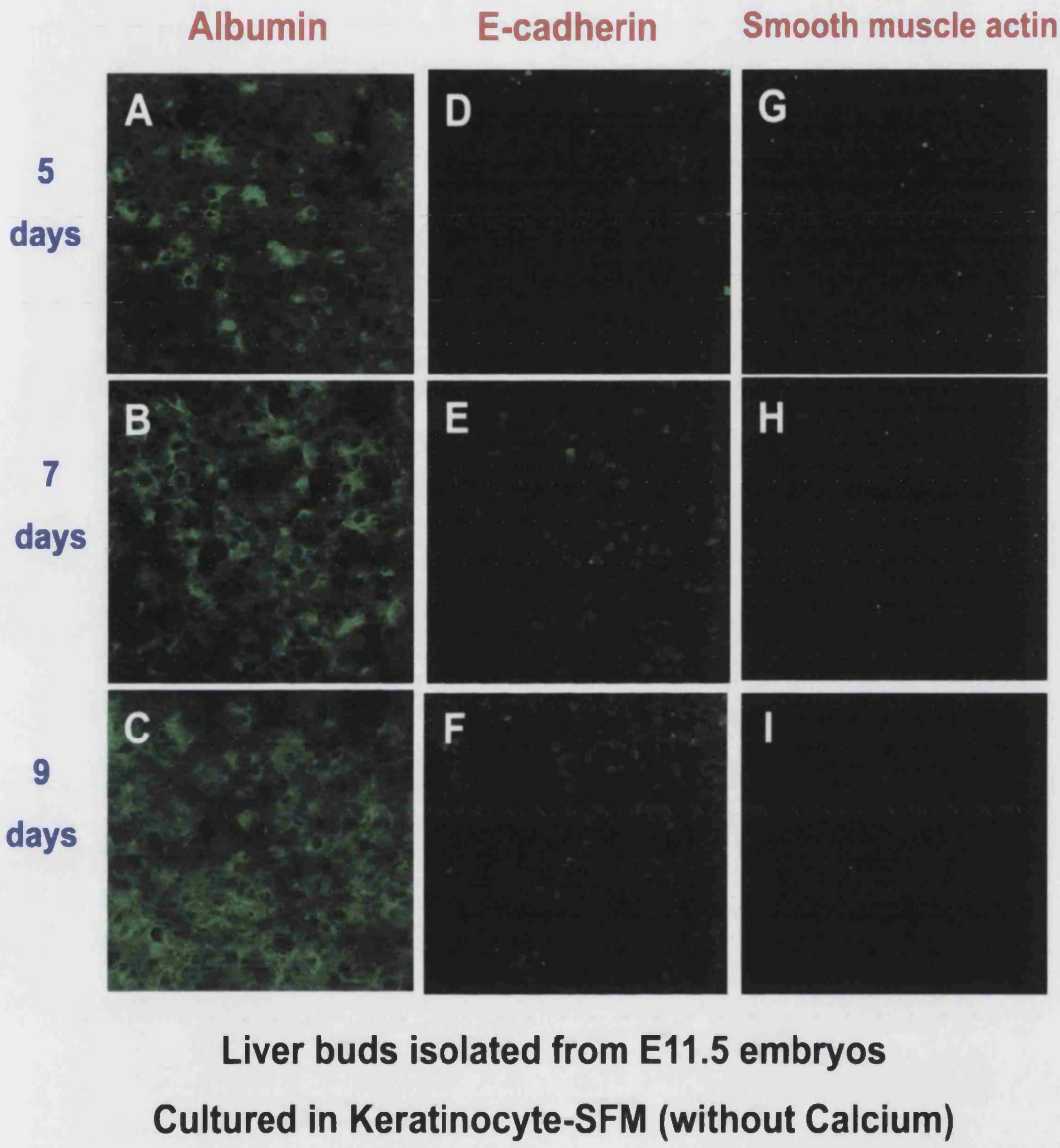


Figure 5.9 Expression of liver markers in KSFM without Ca^{2+}. Liver tissues were immunostained for albumin (A-C), E-cadherin (D-F) and smooth muscle actin (G-I). The liver tissues were isolated from E11.5 mouse embryos and cultured in KSFM without Ca^{2+} for 5, 7 and 9 days on fibronectin-coating coverslips. Scale bar = 20 μm

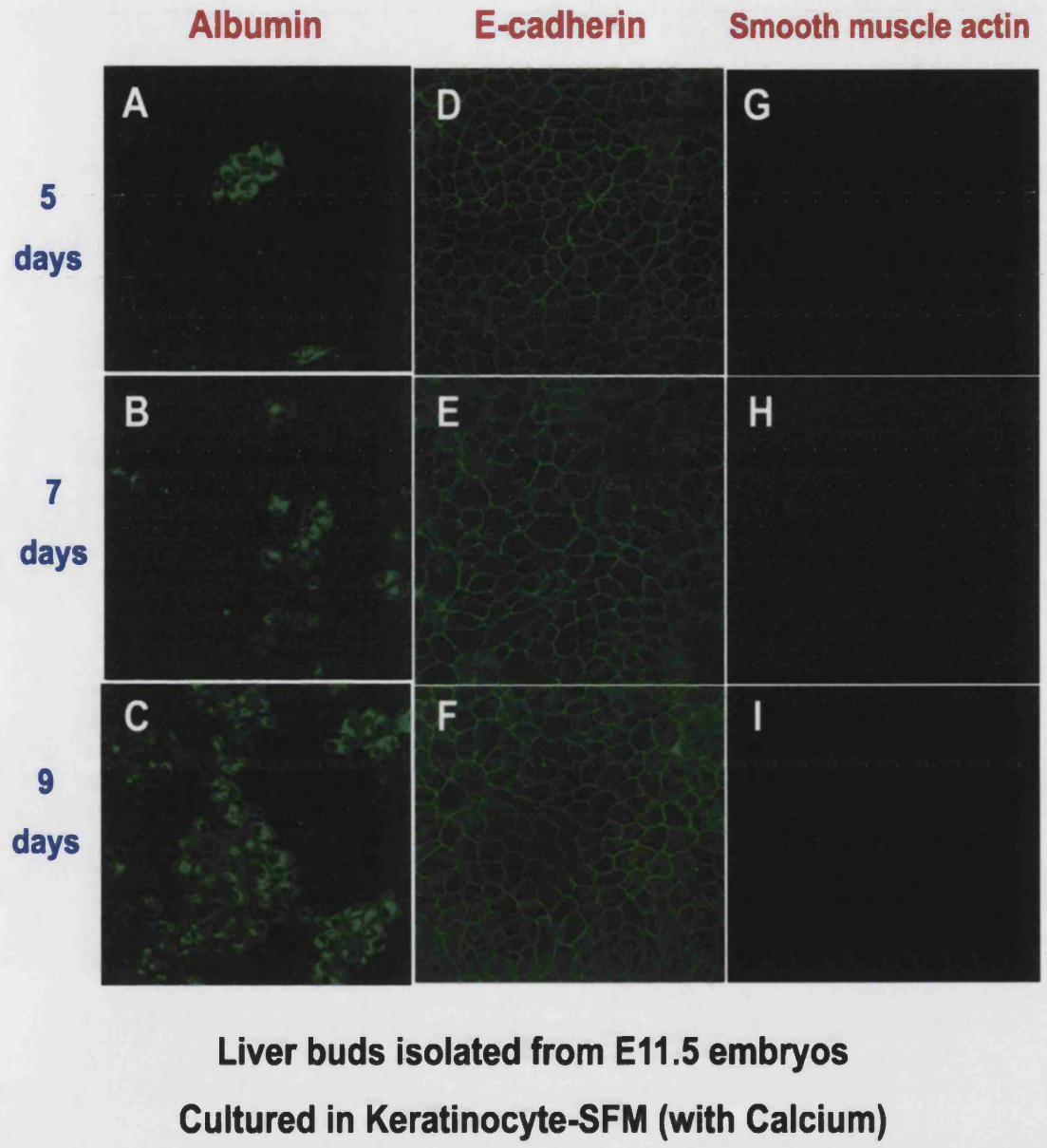


Figure 5.10 Expression of liver markers in KSFM with Ca^{2+}. Immunofluorescence staining of albumin (A-C), E-cadherin (D-F) and smooth muscle actin (G-I) on cultured embryonic liver. The liver tissues were isolated from E11.5 mouse embryos and cultured in KSFM with 0.9mM Ca^{2+} for 5, 7 and 9 days on fibronectin-coating coverslips. Scale bar = 20 μm

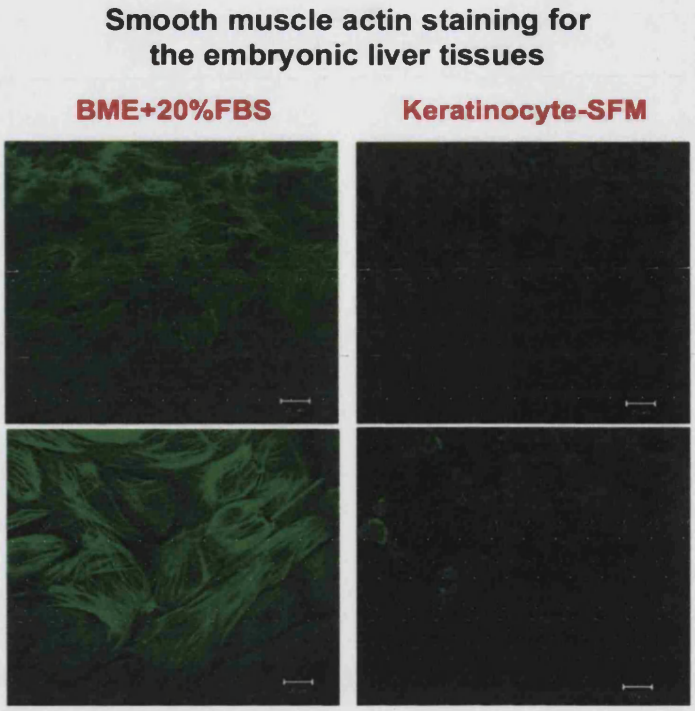


Figure 5.11 Expression of smooth muscle actin (SMA) in isolated liver cultured with BME or KSFM medium. Liver tissue was isolated and cultured for 24hrs in either BME (plus 20% FBS) or KSFM medium. The SMA was expressed in tissue cultured in BME medium but not in KSFM medium. Scale bar = 20 μm

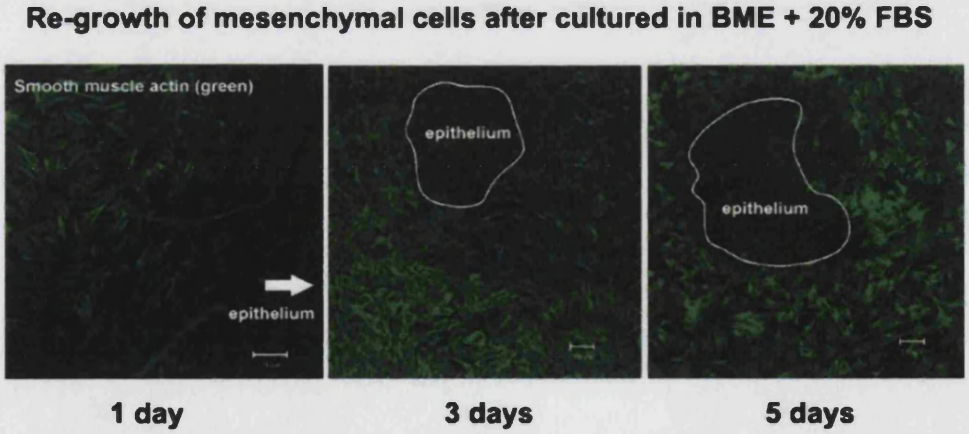
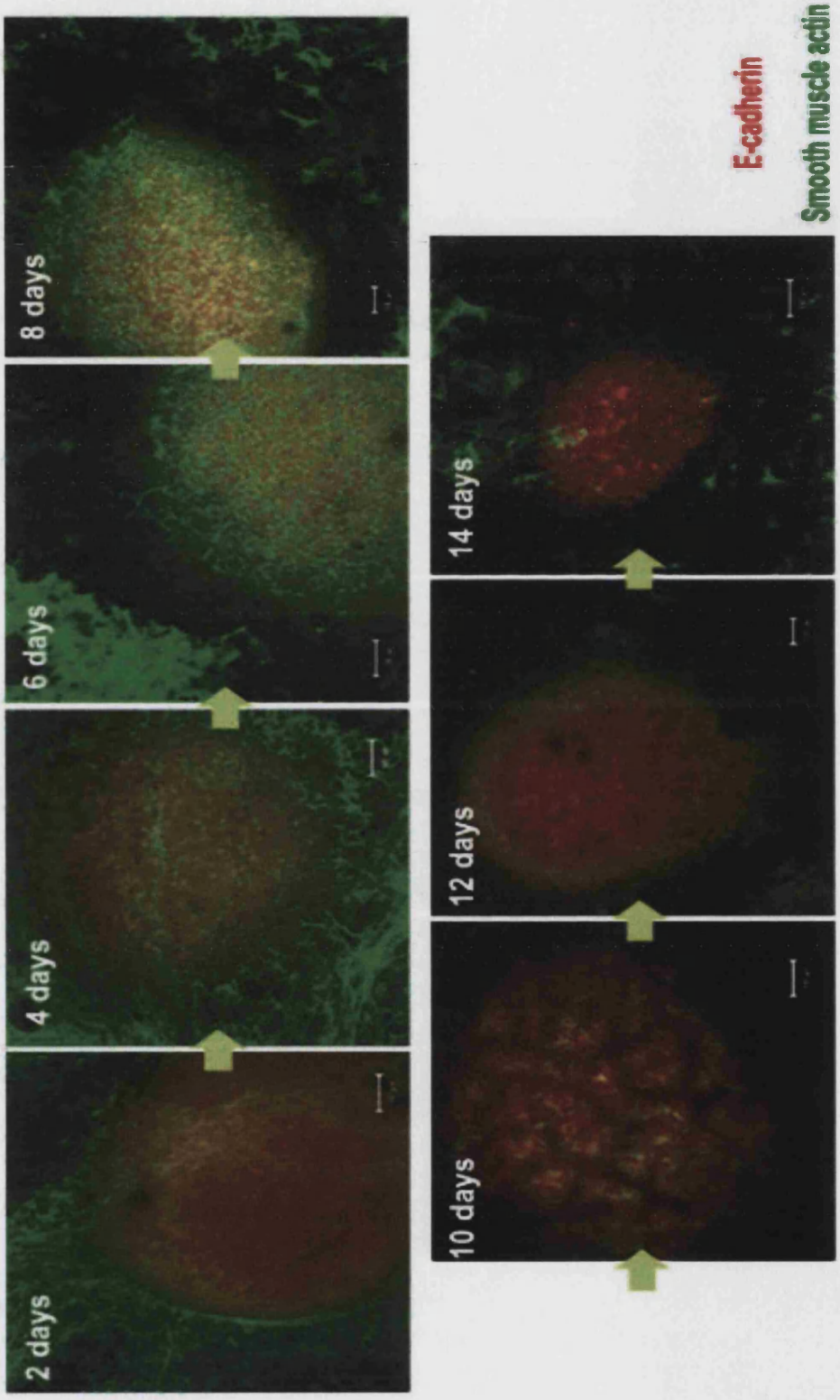


Figure 5.12 Growth of mesenchymal cells in cultured embryonic liver. Embryonic livers were isolated from E11.5 mouse embryo and attached in BME and 20% FBS for 24 hours. The mesenchymal cells were removed using sharp forceps and the remaining tissue was then cultured in BME and 20% FBS for a further 5 days. A sub-population of mesenchymal cells remain and continue to grow and cover the epithelial region when cultured for up to 5 days. Scale bar = 100 μm.



Repression of the growth of mesenchymal cells in KSFM culture medium

Figure 5.13 Repression of mesenchymal cell growth under KSFM culture conditions. The growth of mesenchymal cells is suppressed and moved from the hepatic epithelium during 14-days culture in KSFM medium. The epithelial cells are detected with E-cadherin (red) and mesenchymal cells by smooth muscle actin (green) immunostaining. Scale bar = 100 μm

5.B.3 Optimal culture conditions in KSFM medium

Liver cells cultured in KSFM spread less than when cultured in BME medium (Figure 5.14). This might lead to a higher rate of cell death if the epithelial cells in the centre of the tissue do not receive KSFM. A live-dead cell assay confirmed that there was a higher proportion of dead cells in the centre of the tissue compared to the surrounding area (Figure 5.15). Therefore, to balance cell viability and the efficiency of gene introduction, tests were performed to define the optimal culture conditions for maintenance of epithelial integrity and inhibition of mesenchymal growth. For example, to maintain cell viability, various concentrations of serum was supplemented to the KSFM medium. In this experiment, although the cell viability was improved and the epithelial / hepatic phenotypes were maintained, the mesenchymal cells expanded when more than 5% serum was added to the culture medium (Figure 5.15N). Supplementation of KSFM with a lower concentration of serum (0.5%, 1% and 2.5%) maintained cell viability and the epithelial/hepatic properties. When liver tissue was cultured with KSFM and 1% serum, no growth of the mesenchymal cells was observed (Figure 5.16). As a result, the KSFM + 1% serum was chosen as the "optimal" condition for the remaining experiments. However, the cells did not migrate out and remained massed in the centre of the culture dish.

In order to introduce genes by virus infection a flat culture is necessary. Because the BME medium promotes spreading better than the KSFM, sequential addition of BME (with 20% serum) and KSFM (with 1% serum) was then tested. The aim of this was to achieve the best culture condition for cell growth and ectopic gene expression in epithelial cells. The results suggested that hepatic mesenchymal cells could only be repressed when incubated in KSFM medium for 8 days whereas the

epithelial phenotype was conserved when the cells were incubated in the BME environment for less than 3 days (Figure 5.17& Figure 5.18). Based on these results, the best combination culture condition chosen was: culture in BME (plus 20% serum) for 2-3 days followed by incubation in KSFM (plus 1% serum) for another 8 days (Figure 5.19).

Following this procedure, the efficiency of gene delivery was assessed both by transfection of plasmid DNA and by infection with adenovirus. GFP or DsRed was detected to examine the efficiency of gene delivery into embryonic liver tissues. Although the final culture condition showed the maintenance of the hepatic and epithelial phenotype and reduced mesenchymal contamination (Figure 5.20), the transfection (Figure 5.21) or infection efficiency (Figure 5.20D) into epithelial cells was still extremely low.

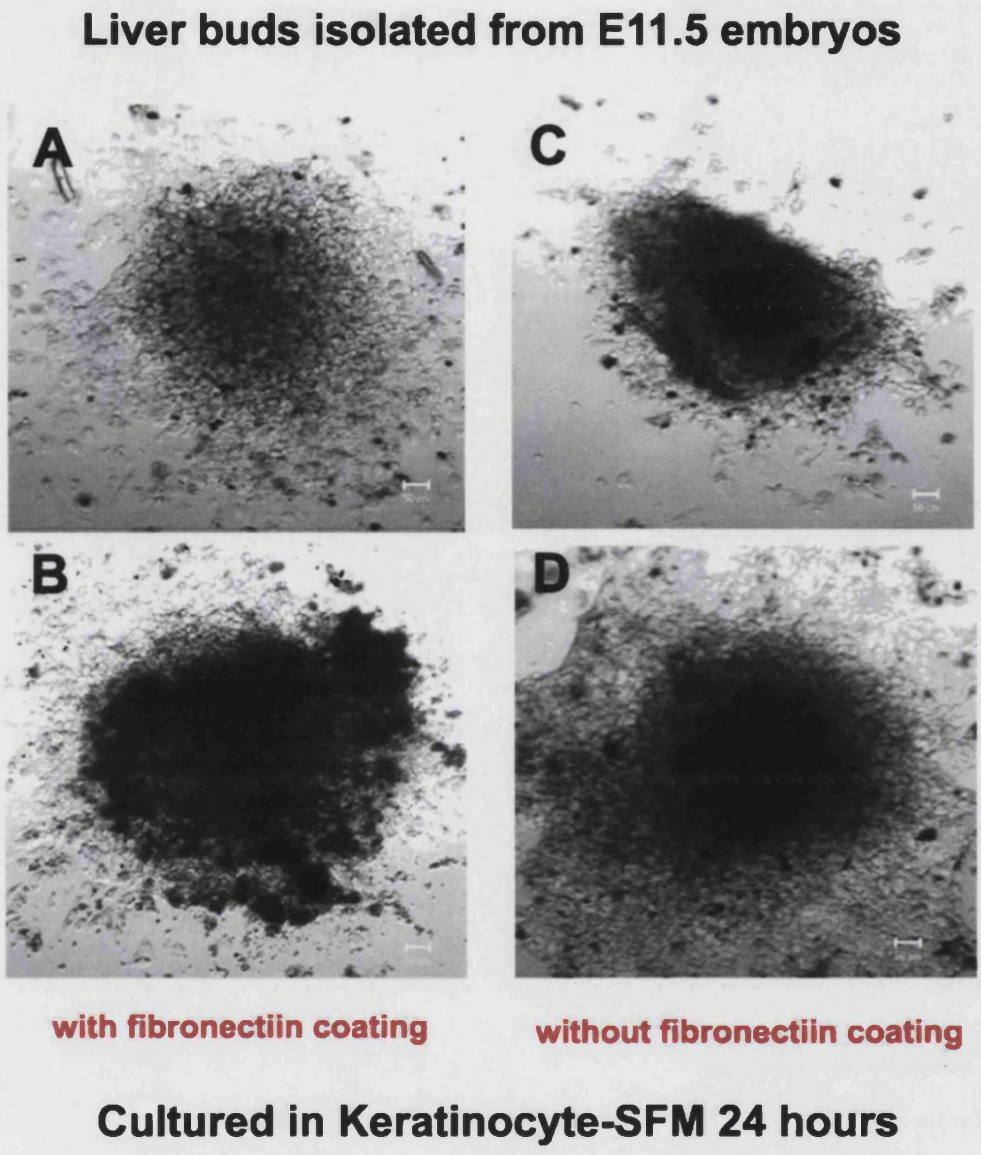


Figure 5.14 Transmitted light images of embryonic liver tissues isolated from E11.5 mouse embryos and cultured for 24hrs in KSFM media. The livers were cultured on coverslips coated with (A,B) or without (C,D) fibronectin. Scale bar = 50 μm

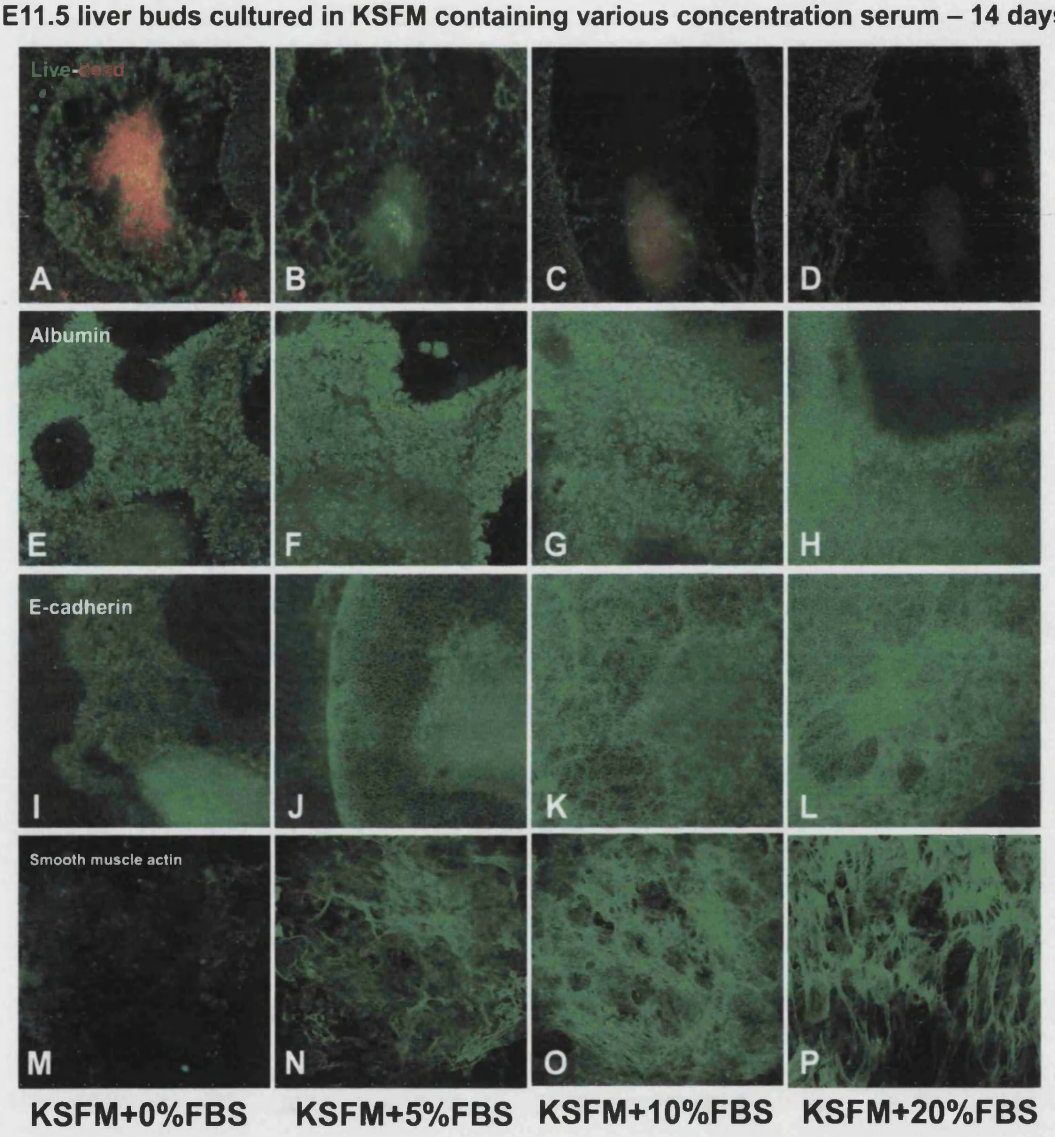


Figure 5.15 The effect of FBS in KSFM on 14-day cultured embryonic liver tissue. The liver tissues were isolated from E11.5 mouse embryos and cultured in KSFM supplemented with 0%, 5%, 10% and 20% of FBS. The cell viability (A-D) and the expression of albumin (E-H), E-cadherin (I-L) and smooth muscle actin (M-P) was then examined. Scale bar = 20 μm

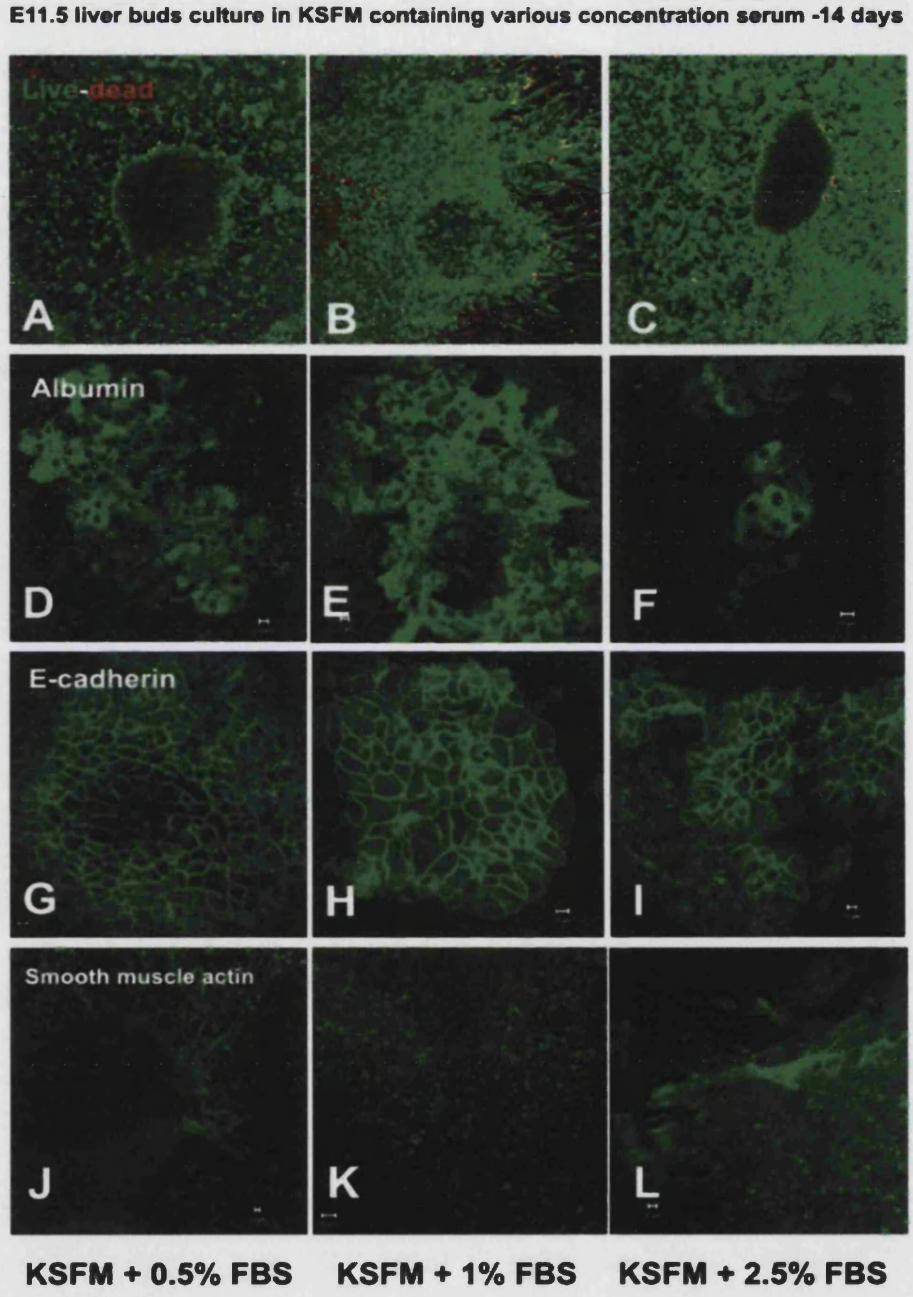


Figure 5.16 The effect of low concentration of FBS in KSFM on 14-day cultured embryonic liver cells. The liver tissues are isolated from E11.5 mouse embryos and cultured in KSFM supplemented with 0%, 1% and 2.5% of FBS. The cell viability (A-C) and the expression of albumin (D-F), E-cadherin (G-I) and smooth muscle actin (J-L) was then examined. Scale bar = 20 μm

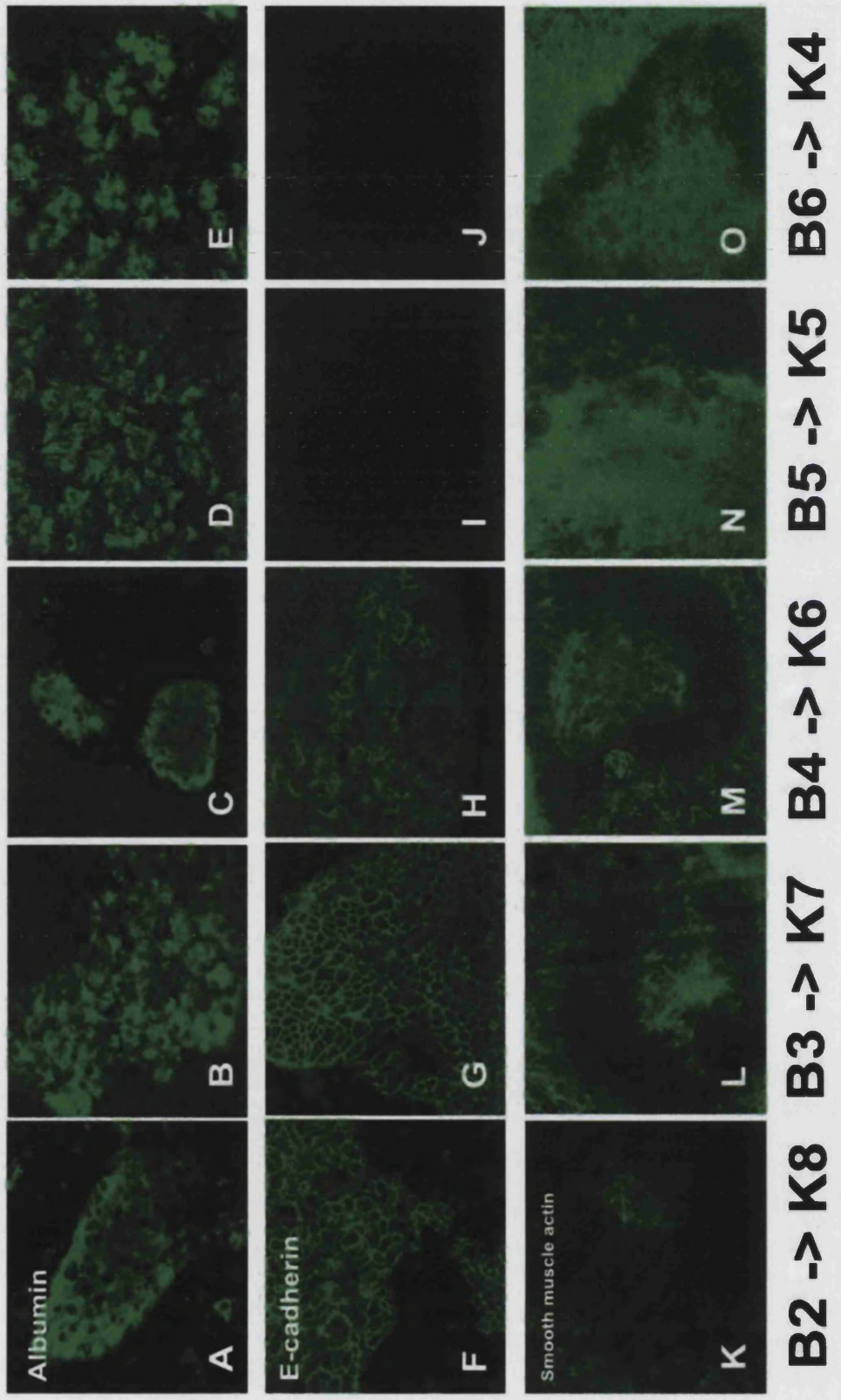

E11.5 liver culture in BME & KSFM -total 10 days
Albumin
A
B
C
D
E
E-cadherin
F
G
H
I
J
Smooth muscle actin
K
L
M
N
O
B2 -> K8
B3 -> K7
B4 -> K6
B5 -> K5
B6 -> K4

Figure 5.17 Incubation period test for the combination of BME and KSFM medium-1. Liver tissues were isolated from E11.5 mouse embryos and cultured in BME and 20% FBS and then transferred into KSFM plus 1% FBS for different time periods (total culture time was 10 days). The expression of albumin (A-E), E-cadherin (F-J) and smooth muscle actin (K-O) were examined by immunofluorescence staining.

E11.5 liver culture in BME & KSFM - in KSFM 8 days
Albumin
A
B
C
D
E
E-cadherin
F
G
H
I
J
Smooth muscle actin
K
L
M
N
O
B2 -> K8
B3 -> K8
B4 -> K8
B5 -> K8
B6 -> K8

Figure 5.18 Incubation period test for the combination of BME and KSFM medium-2. Liver tissues were isolated from E11.5 mouse embryos and cultured in BME plus 20% FBS and then transferred into KSFM with 1% FBS for 8 days. The expression of albumin (A-E), E-cadherin (F-J) and smooth muscle actin (K-O) was examined by immunofluorescence staining.

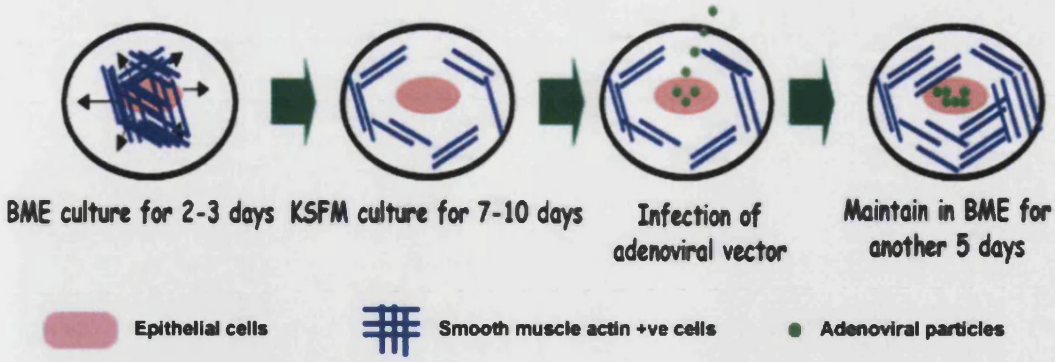


Figure 5.19 A working model of gene delivery into embryonic liver cells using KSFM culture medium.

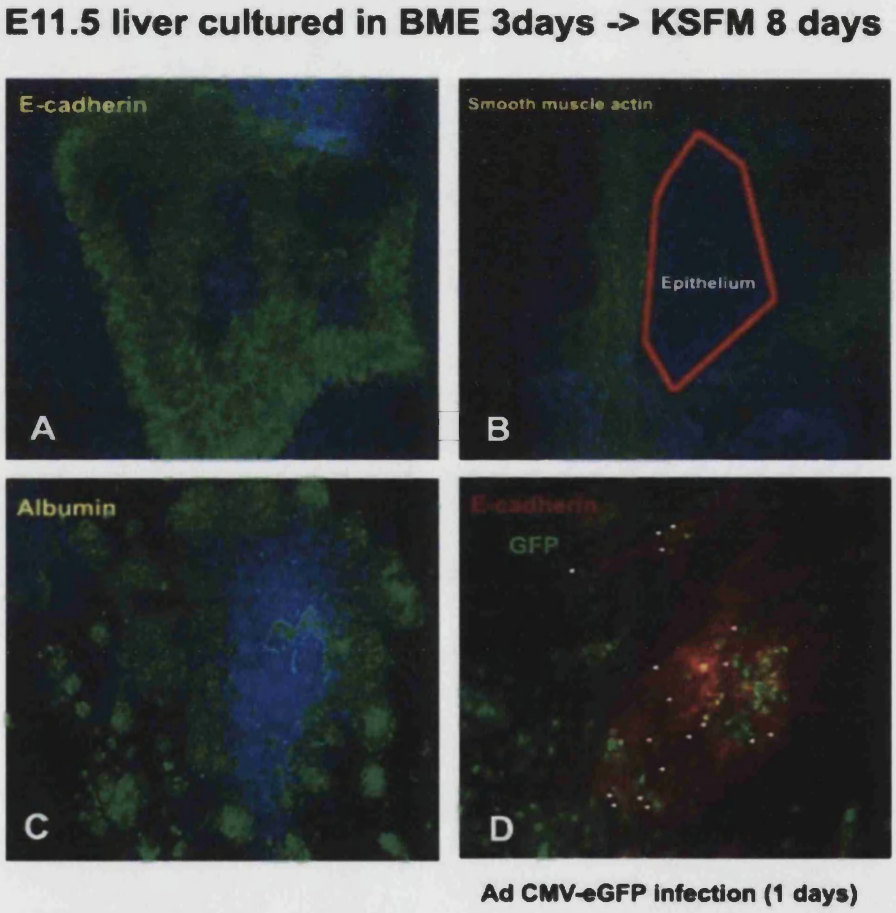


Figure 5.20 Examination of liver, epithelial and virus infection using optimal culture condition in embryonic liver tissues. The examination of E-cadherin (A), smooth muscle actin (B), albumin (C) (green; DAPI stating was shown in blue) and adenoviral infection efficiency (D) in cultured embryonic liver tissue. Livers were cultured in BME plus 20% FBS for 3 days and then the media was changed to KSFM with 1% FBS for a further 8 days. In (D), Ad CMV-eGFP infection efficiency of the epithelium was assessed by staining for E-cadherin (red) and GFP (green).

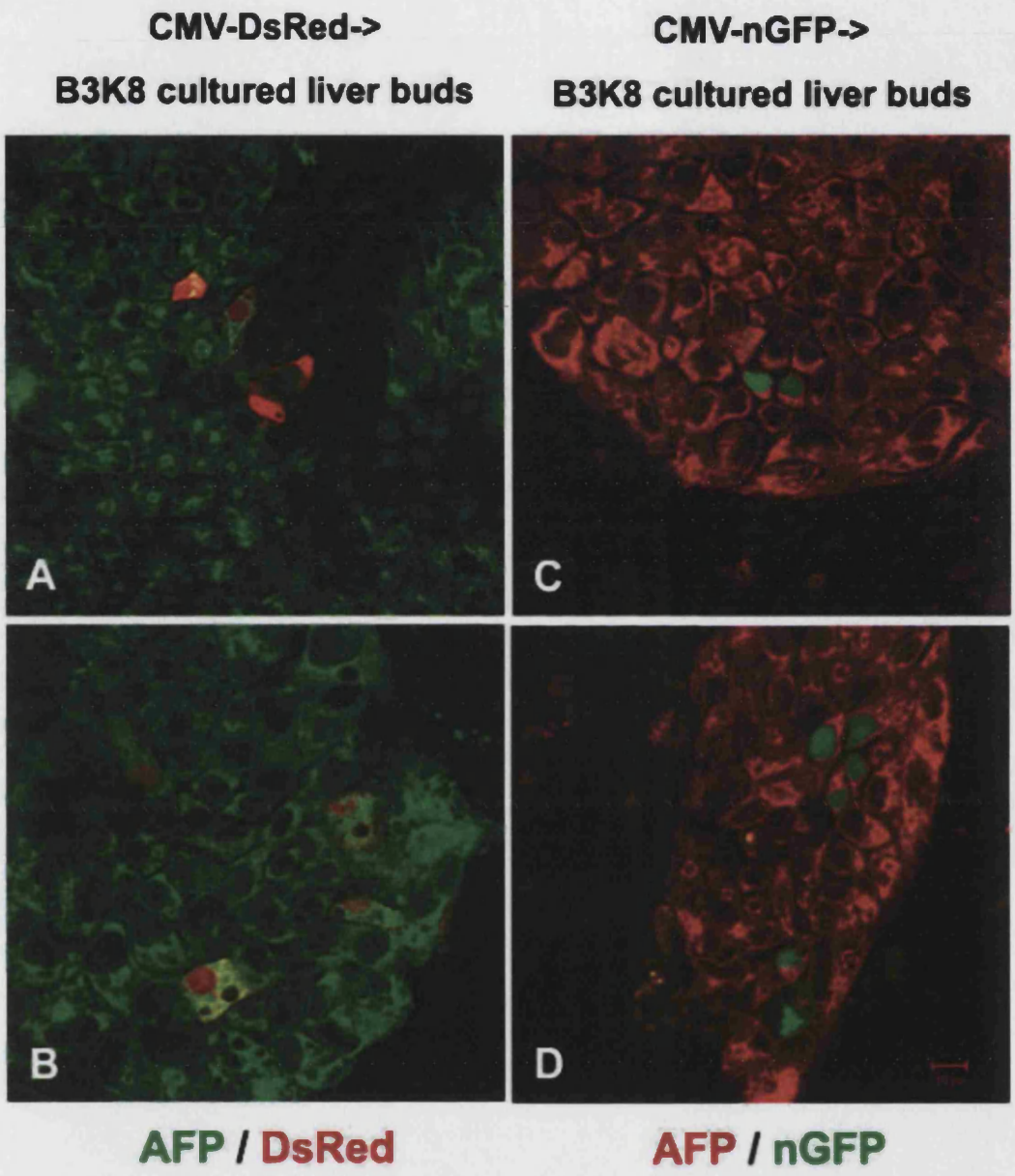


Figure 5.21 Transfection of embryonic liver cultures. The embryonic liver cells from E11.5 embryos were cultured with the same procedure as described in Figure 5.20 and then transfected with 2μg plasmid DNAs of CMV-DsRed (A,B) or CMV-nGFP (C,D). The detection of AFP (green) and DsRed (red) (A,B) or GFP (green) and AFP (red) (C,D) was performed after 24 hours of transfection. Scale bar = 10 μm

5.C Discussion

Embryonic cells may possess greater cellular plasticity than differentiated cells. Based on this idea it might be easier to direct embryonic cells to a new cell fate (Efrat, 2002). In liver, several markers for progenitor cells were found in the last few years. They include serum protein α1-fetoprotein and the intermediate filament protein CK14 (Shafritz and Dabeva, 2002).

We tried to build a suitable embryonic liver culture model to test whether (i) ectopic genetic expression is possible and (ii) Pdx1 can convert liver to pancreas. Although the embryonic liver tissues can be cultured in BME and KSFM medium it is not possible to add genes to the cultures with the methods available. It suggested that it is very difficult to clearly separate the epithelial from the mesenchymal cells in embryonic liver tissue and, on the other hand, again confirms the importance of mesenchymal cells for liver development (Cascio and Zaret, 1991). To further refine the current system, the combination of mechanical manipulation and the utilisation of selective culture medium might be considered.

In brief, the results from this chapter shows that the embryonic liver cells seemed to be unsuitable to culture in either BME or KSFM culture medium. However, the fact that the growth of mesenchymal cells was inhibited under KSFM culture conditions provides an alternative means to enrich the epithelial cell culture from other hepatic-like cells. Trials on the inoculation of adult hepatocytes in KSFM are discussed in the next chapter.

Chapter 6.
Long-term culture of rat hepatocytes & conversion into pancreatic-like cells

Overview

In Chapter 5, a culture system for mouse embryonic liver tissue was produced but this requires further refinement. In the present chapter, we sought to extend further the use of KSFM culture medium to primary adult rat hepatocytes. Specifically, we aim to develop a suitable *in vitro* culture system for long-term culture of primary hepatocytes. First, a broad introduction to the principles underlying isolation and culture of hepatocytes from adult rodents and the current obstacles to the long-term maintenance of mature hepatocytes will be introduced. Afterwards, results will be presented showing that KSFM can maintain the expression of liver proteins in rat hepatocytes for up to 28 days. In addition, functional experiments on hepatocytes cultured in KSFM will be performed to confirm that the function of specific pathways is maintained in long-term cultured hepatocytes. Finally, the question of cell plasticity between liver and pancreas was addressed using this culture system and adenoviral infection technique. Preliminary results suggested the partial transdifferentiation of differentiated liver cells into pancreatic cells.

6.A Introduction

6.A.1 Isolation of adult hepatocytes

The liver has a multitude of functions including the regulation of carbohydrate, fat and protein metabolism, the detoxification of endo- and xenobiotics, and the synthesis and secretion of plasma proteins and bile. The materials for studying liver functions include isolated perfused liver (Berry et al., 1992), liver slices (Parrish et al., 1995) or freshly isolated and cultured hepatocytes (Mitaka, 1998) (Table 6.1). Among them, the isolation and culture of hepatocytes from adult animals is the most common method to

be used. The technique for isolation of hepatocytes now widely used is based on the two-step collagenase perfusion technique first developed by Berry and Friend (Berry and Friend, 1969). As well as enzymatic digestion, different non-enzymatic mechanical separation methods were used to obtain liver cells (Schreiber and Schreiber, 1973). These include liver homogenisation, trituration of liver samples, and filtration of the tissue through fine sieves. However marked cellular damage was observed under all conditions. Besides vigorous physical breakdown of the liver, a range of digestive enzymes were tested for their potential to acquire intact cells. For example, trypsin, pronase and lysozyme were all used to disperse liver tissue into a single cell suspension (Howard et al., 1967; Seglen, 1976) but these enzymes did not produce large numbers of viable hepatocytes. By the late 1980s collagenase was used as a successful dissociating agent (Berry and Friend, 1969; Klaunig et al., 1981).

To date, the two-step collagenase perfusion technique is the method of choice for the isolation of large numbers of viable hepatocytes from adult animals. In the first step, a calcium-free medium containing a calcium chelator is perfused through the liver. Removal of calcium ions (by EDTA, EGTA or citrate) from epithelial cells results in the rapid destruction of intercellular junctions and therefore loosening of the cell-cell contacts(Troyanovsky, 2005). The second step is the introduction of the enzyme collagenase into the liver lobes. The procedure is carried out *in situ* using the animals' own circulatory system to perfuse the liver via the portal vein or *vena cava* (retrograde perfusion). It has previously been shown that the two-step collagenase isolation procedure can yield a high percentage of viable liver cells. Seglen proposed several experiments to assess the importance of the following factors for hepatocyte isolation (Seglen, 1976).

(A) ***Ca^{2+}:*** Ca^{2+} plays a dual role in the hepatocyte isolation protocol. On the one

hand, removal of Ca^{2+} is essential to disrupt the Ca^{2+}-dependent E-cadherin molecules between adjacent hepatocytes and hence improve both the quantity and quality of dispersed hepatocytes. The presence of Ca^{2+} is also required for the activity of collagenase (1-5 mM of Ca^{2+} is sufficient to sustain collagenase activity for liberation of hepatocytes) (Berry et al., 1997; Takahashi and Seifter, 1970). Regarding these properties of Ca^{2+}, both the concentration and timing of re-introduction of Ca^{2+} back into liver during the hepatocyte preparation process are critical for obtaining intact cells. Seglen demonstrated that Ca^{2+} must first be removed from the tissue by pre-perfusion with EGTA or EDTA containing medium then added back into the perfusion medium with collagenase to obtain a high quantity of intact, viable cells (Seglen, 1976).

(B) ***Concentration and quality of collagenase***: Collagenase is a key element for hepatocyte isolation. It is therefore necessary that the user tests the activity of each batch of collagenase. Different batches of collagenase exhibit differing efficiencies during preparation of hepatocytes (Howard et al., 1973). Seglen showed that crude collagenase and purified collagenase displayed similar activity on liver cell dispersion in the presence of same concentration of calcium ions (Seglen, 1976). In addition, to obtain the best quality and quantity of dispersed hepatocytes, Klaunig et al suggested using 100U/ml of type I collagenase as the concentration of choice for mouse hepatocyte preparation(Klaunig et al., 1981).

(C) ***pH and buffering:*** The pH value of the perfusion medium should be kept at approximately pH 7.4. The detection of a rapid drop in pH during perfusion is due either to the enzymatic breakdown of glucose in the medium or the generation of bicarbonate from the dissolution of carbon dioxide in water. Therefore a strong buffering system is essential to ensure healthy cells. Several buffering agents have been suggested to maintain the pH. Among the buffers, the addition of HEPES into

the perfusion medium has proved to be the simplest and most practical (Seglen, 1976).

(D) ***Other factors:*** In addition to the points raised above, the production of isolated hepatocytes can also be affected by other factors, such as the perfusion flow rate (optimum of 5-10ml/minute for mouse, 25-30 ml/minute for rat) or the force of centrifugation following isolation (optimum of 50-100 *g* for liver cell isolation).

After the liberation of hepatocytes, monitoring of cell viability should be performed. Several techniques for viability testing have been suggested and most depend on an intact cell membrane. Examples include trypan blue exclusion, measurement of ATP content (Olinga et al., 1998), detection of the reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (so called MTT test) (Denizot and Lang, 1986), monitoring of leaking lactate dehydrogenase or detection of cell-specific biomarkers such as aminotransferase (AST and ALT) or total bilirubin (Behrsing et al., 2003).

	Hepatocyte suspension	Liver slices	Hepatocyte Cultures
Pros	1. Standard preparation protocol 2. Acquirement of large number of hepatocytes 3. Similar response to *in vivo* pharmacological and toxicological effect	1. Relatively quick and easy to prepare 2. Preservation of liver architecture and includes all liver cell types 3. Possibility for cryopreservation	1. Extended survival periods 2. Standard preparation protocol 3. Possibility for lengthy and complex experiments
Cons	1. Disruption of intercellular contacts & cell communications 2. Loss of cell polarity and shape 3. Damage to receptors 4. Short survival periods (~few hours)	1. Short survival period (~ few days) 2. Impaired preservation of metabolizing enzymes (e.g. cytochrome P450) 3. Slower metabolising rate for certain metabolites	1. Contamination by non-hepatic cells 2. Loss of hepatic phenotype (e.g. metabolizing enzymes, transportation systems) 3. Time consuming (e.g. concentration of additives in the medium should be tested prior to experiment)

Table 6.1 Comparison of *in vitro* systems for studying liver function (Berry et al., 1992; Cervenkova et al., 2001; Denizot and Lang, 1986; Sidelmann et al., 1996)

6.A.2 Limitations of currqw ent *in vitro* hepatocyte culture systems

Hepatocytes in suspension show rapid loss of their differentiation status after plating out onto the culture dish (Agius, 1987). Dedifferentiation is reflected not only in decreased liver-specific functions, but there is also an alteration of morphology: the cells flatten, depolarize, and lose many of the surface characteristics of normal hepatocytes *in vivo*. The mechanisms responsible for loss of differentiated properties presumably include downregulation of transcription factors involved in liver specific gene expression (Padgham et al., 1993). The loss of differentiated functions has been attributed to the change in environmental conditions (extracellular matrix, hormonal conditions) following cell isolation (Padgham and Paine, 1993) and is most apparent in the rapid decline of total cytochrome P450s (CYPs), particularly the CYP3A1 isoform (Padgham et al., 1993). Therefore, after a standard hepatocyte isolation protocol was established, attempts were made to produce suitable culture conditions for hepatocytes. Studies of liver function are generally confined to the first few days of culture but this precludes longer-term studies. Conventional approaches to maintaining the differentiated properties of isolated hepatocytes in culture include supplementation of the medium with hormones such as dexamethasone (Agius et al., 1986; Enat et al., 1984), co-factors such as nicotinamide, pyruvate, DMSO and phenobarbital (LeCluyse et al., 1996; Waxman et al., 1990); the application of extracellular matrix components (Bissell et al., 1987; Dunn et al., 1989; Gomez-Lechon et al., 1998) and co-culture with non-parenchymal epithelial cell-types (Agius, 1988; Rogiers and Vercruysse, 1993). Recently, Mitaka has compiled an extensive review on the development of conditions for maintenance of hepatocytes in culture (Mitaka, 1998). Although most groups declared that they had successfully

found a suitable medium for human or rodent hepatocyte culture, only a limited number of liver-specific functions were demonstrated in individual studies. So, there is still no consensus on the best conditions for culturing isolated hepatocytes (Table 6.2) and this will ultimately depend on the experimental procedures to be performed on the hepatocytes.

Year	Authors	Medium	Supplements
1995	Kojima et al.	L-15	0.2% BSA, 20mM HEPES, 0.5mg/L insulin, 10^{-7}M dexamethasone, 5.5mM galactose, 30mg/L proline, 20mM $NaHCO_3$, 5mg/L transferrin, 0.2mg/L $CuSO_4.5H_2O$, 0.5mg/L $FeSO_4.4H_2O$, 0.75mg/L $ZnSO_4.7H_2O$, 0.05mg/L $MnSO_4$, 5 μg/L Na_2SeO_3, 10ng/ml EGF, antibiotics
1998	Miyazaki et al.	DMEM	2g/L BSA, 2.25g/L glucose, 2g/L galactose, 0.1g/L ornithine, 0.03g/L proline, 0.305g/L nicotinamide, 0.544 mg/L $ZnCl_2$, 0.75mg/L $ZnSO_4.7H_2O$, 0.2mg/L $CuSO_4.5H_2O$, 0.025mg/L $MnSO_4$, 146.1mg/L glutamine, 1g/L ITS, 10^{-7}M Dex, 40ng/ml HGF, 20ng/ml EGF, antibiotics
1999	Lilja et al.	Ham's F12 medium: WE =1:1	0.25% bicarbonate, 15mM HEPES, 65.5 μM ethanolamine, 100 μg/ml trnaferrin, 0.6g/L insulin, 10^{-7}M dexamethasone, 10nM glucagon, 32.25mg/L proline, 7.18 μM linoleic acid, 7mM glucose, 0.4mM sodium pyruvate,0.1mM ascorbic acid, antibiotics
2000	Runge et al.	DMEM(no glucose): DMEM(with glucose): MEM=1:1:2	1g/L albumin, 5.5mM galactose, 0.5mM glutamine, 0.3mM ornithine, 0.13 mM proline, 5mM HEPES, 2.5ng/ml Na-selenite, 2.5 μg/ml transferrin, 0.2mM $ZnCl_2$, 0.1mM $MnSO_4$, 2.75 μg/ml insulin, 50nM dexamethasone, 40ng/ml HGF, 20ng/ml EGF, antibiotics
2001	Mizuguchi et al.	DMEM	10%FBS, 20mM HEPES, 10mM nicotinamide, 1mM ascorbic acid-2-phosphate, 10^{-7}M dexamethasone, 1mg/ml galactose, 30 μg/ml proline, ITS, 10ng/ml EGF, antibiotics
2002	Katsura et al.	WEM / KSFM (contain FBS or human serum)	10mM nicotinamide, 30mg/L proline, 1mM ascorbic acid 2-phosphate, 0.5 μg/ml insulin, 10^{-7}M dexamethasone, 10ng/ml EGF, antibiotics
2003	Iocca et al.	DMEM-F12 based	2%DMSO, 1 μM [+]-alpha-tocopherol, 25ng/ml EGF

2004	Vanchaecke et al.	DMEM	0.5U/ml insulin, 7ng/ml glucagon, 1% antibiotics, 10%FBS
2005	Gardmo et al.	WE+Glutamax Or Weymouth 752 medium	10nM insulin, antibiotics
2005	Siendones et al.	WE	1 μ M insulin, 0.6 μ M hydrocortisone, 15mM HEPES, 2mM glutamin, 26mM NaCO3, antibiotis
2005	Noreault et al.	WE	10^{-7}M dexamethasone, 10^{-8}insulin, 10^{-7}sodium seletine, 0.3mM ascorbate
2005	Li et al.	RPMI-1640	10mM HEPES, 1% ITS, 1 μ M dexamethasone, antibiotics
2005	Kimura et al.	Ham's nutrient mixture F-10	1nM dexamethasone, antibiotics
2005	Abdelmegeed et al.	Modified Chee's medium	2mM L-glutamine, 6.25 μ g/ml tranferrin, 0.1 μ M dexamethasone, 1 μ M insulin,
2005	Schmidt et al.	DMEM	20%FBS, 0.1 μ M dexamethasone, antibiotics

Abbreviations - DMEM: Dulbecco's Modified Eagle's Medium; L-15: Leibovitz L-15 Medium; MEM: Minimum essential medium; WE: Williams' Medium E; KSFM: Keratinocyte-stimulating factor medium, ITS: Insulin-transferrin-selenium mixture; EGF: Epidermal growth factor; HGF: Hepatocyte growth factor; BSA: Bovine serum albumin; FBS: Foetal bovine serum

Table 6.2 Summary of media used for hepatocyte culture (selected publications from 1995-2005). Antibiotics referred to were penicillin and streptomycin.

6.B Development of a serum-free culture system for the maintenance of hepatic differentiation phenotype

6.B.1 Preservation of hepatic markers in KSFM medium

Despite numerous attempts, it is difficult to maintain hepatocytes in a well-differentiated state for more than a few days. Therefore it was sought to develop a protocol for maintenance of hepatic functions *in vitro* by addition of a simple, well-defined medium. Based on the previous observation in embryonic liver culture (see Chapter 5) and the use of KSFM in the culture of epithelial cells by several groups (Andl et al., 2003; Chen et al., 2002; Vallette et al., 1998). The potential of KSFM as a medium for the culture of primary hepatocyte cultures was then tested.

Adult rat hepatocytes isolated by the collagenase perfusion technique were plated either on glass coverslips or on tissue culture plastic (see chapter 2 for further details). The cells were allowed to attach in Williams' Medium E (WE) containing 10% serum and maintained for 6-10 hours before changing to serum-free defined WE or KSFM medium (see transmitted images in Figure 6.1). Initial results based on immunofluorescence detection and Western blotting were encouraging. For the same culture period (up to 72 hours), albumin protein expression was preserved in cells maintained in KSFM but not in WE medium (Figure 6.2). A similar result was also observed for transferrin expression (Figure 6.3).

The expression of hepatic proteins other than albumin and transferrin was compared between WE and KSFM culture medium with the indicated supplements

(see section 2.B.2.3). The liver proteins included serum proteins α1-antitrypsin (Figure 6.4), haptoglobin (Figure 6.5), liver enriched transcription factors C/EBPα (Figure 6.6), C/EBPβ (Figure 6.7), HNF4α (Figure 6.8), RXRα (Figure 6.9), enzymes associated with ammonia detoxification: glutamine synthetase (GS) and carbamylphosphate synthetase (CPS) (Figure 6.10), the Phase II metabolising enzyme UDP-glucuronosyltransferase (UGT) (Figure 6.7) and the epithelial marker E-cadherin (Figure 6.11). The results showed that all the proteins examined were maintained in KSFM-cultured conditions in comparison to WE culture media. Connexin 32 (Cx32) and connexin 26 (Cx26) are the two main gap junction proteins expressed in hepatocytes(Kumar and Gilula, 1986; Nicholson et al., 1987). Gap junction intracellular communication plays an important role in regulating cell survival and apoptosis (Krysko et al., 2005), cell differentiation and proliferation (Cheng et al., 2004) and tumourigenesis (Luebeck et al., 2005). Therefore, the expression of Cx32 was also examined. The expression of Cx32 was only detected in hepatocytes cultured in KSFM with EGF, BPE in the absence and presence of dexamethasone (Figure 6.12).

Western blotting for several hepatic proteins including albumin, transferrin and HNF4α was also carried out. The expression of proteins from hepatocytes cultured under different conditions for 24 and 72 hours were examined. The protein expression was sustained in KSFM culture medium, the best in KDS condition, compared to those WE medium (Figure 6.13). This result confirmed in semi-quantitative terms, the immunostaining results.

Based on the above observations, it was investigated whether KSFM could prevent de-differentiation of cultured hepatocytes.The expression of vimentin, a cytoskeletal de-differentiation marker (Blaheta et al., 1998), after 72 hrs of culture was examined. Vimentin was expressed in rat hepatocytes cultured in WS and WDS but not in cells cultured in KS and KDS (Figure 6.14a). It is worth noting that two different

groups of vimentin positive cells were found in WS cultured cells after 72 hours: individual vimentin-positive cells were found scattered (Figure 6.14b) as well as in groups (Figure 6.14c). Following inoculation of the cells, some attached as individuals whereas others attached in groups and both undergo de-differentiation after culture in WS for 3 days. Altogether, these observations suggested that KSFM may play a role in the preservation of the differentiation state of the isolated rat hepatocytes.

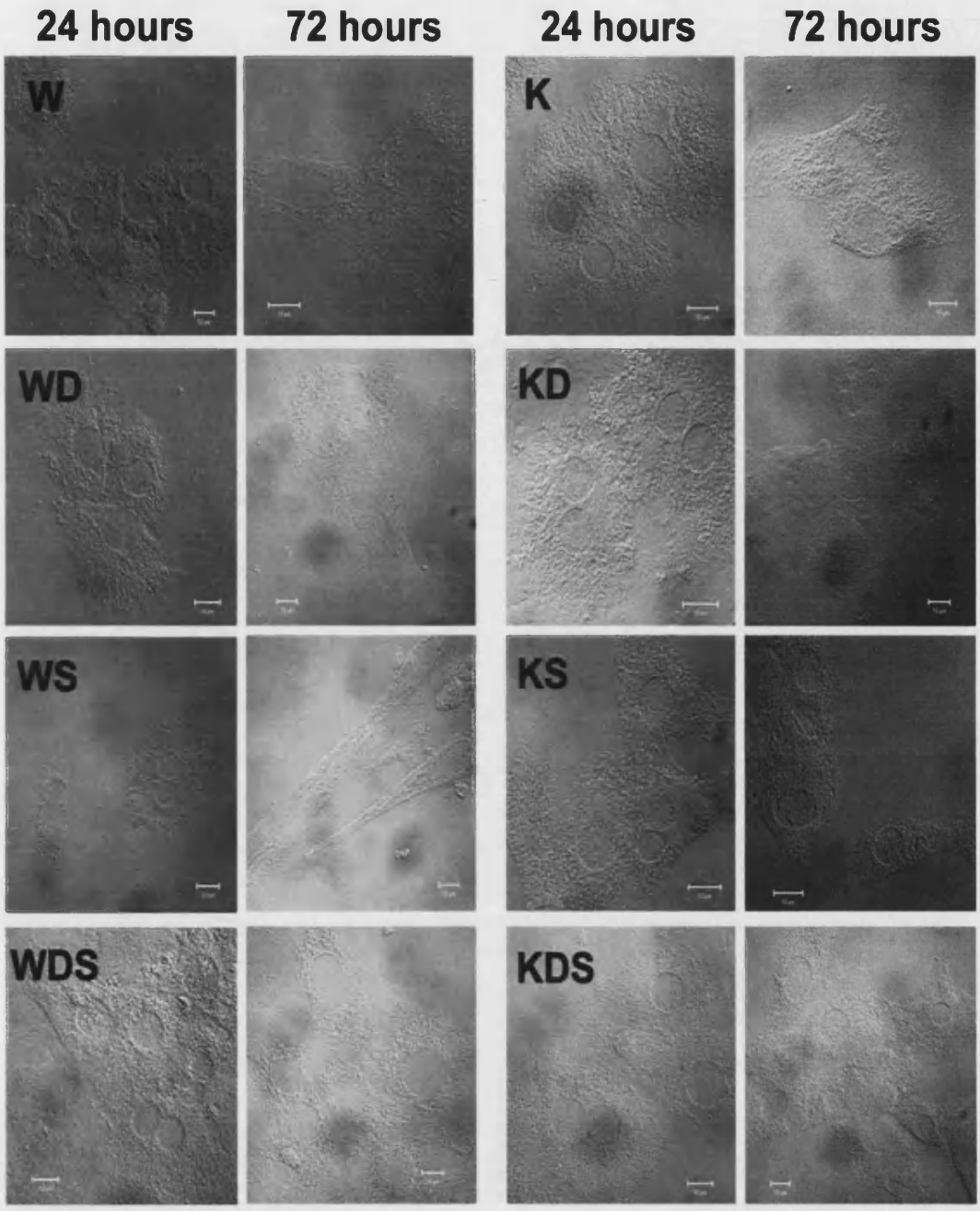
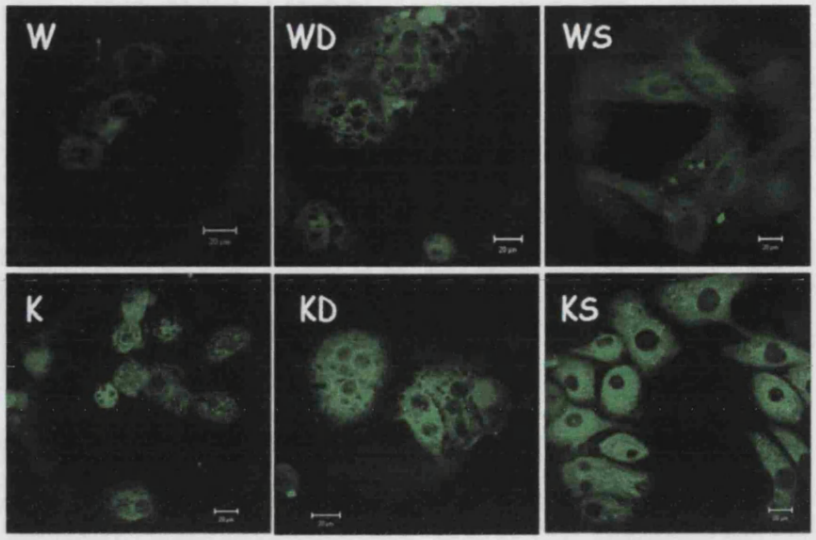
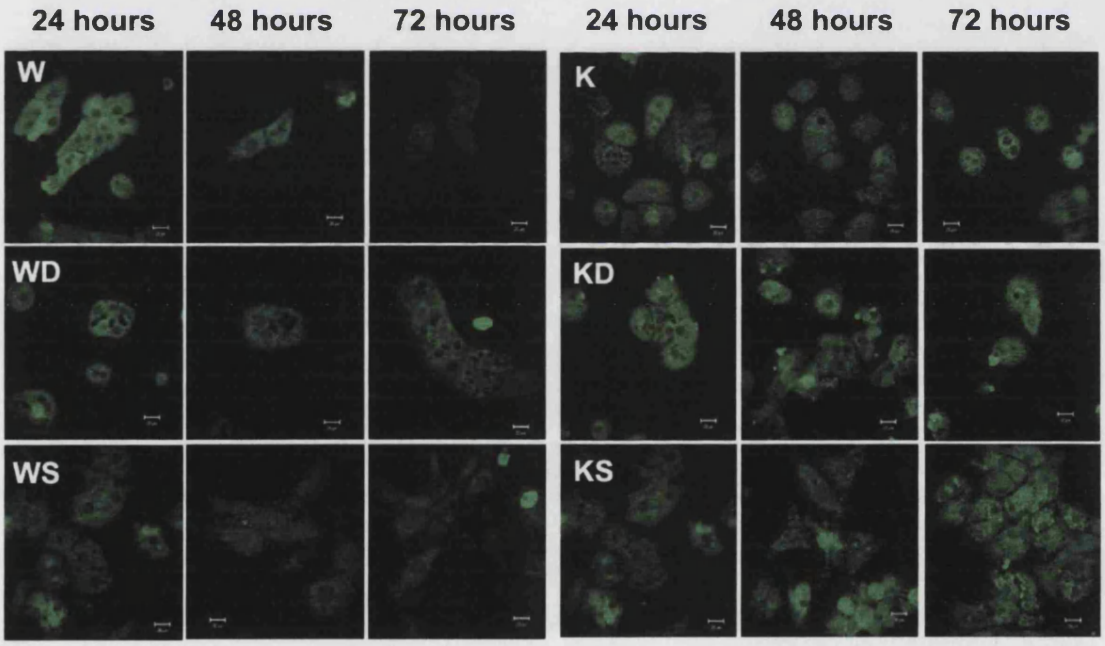


Figure 6.1 Transmitted light images of isolated rat hepatocytes maintained in culture for 24 or 72 hours in either WE or KSFM medium with the indicated supplements. Scale bar = 10 μm. For abbreviations, see section 2.B.2.3.



Immunostaining for albumin

Figure 6.2 Albumin expression in isolated rat hepatocytes cultured for 72 hours in different culture media. The preservation of albumin is observed in hepatocytes cultured in KSFM medium. Scale bar = 20 μm. For abbreviations, see section 2.B.2.3.



Immunostaining for transferrin

Figure 6.3 Transferrin expression in isolated rat hepatocytes cultured for 24, 48 and 72 hours in different culture media. Scale bar = 20 μm. For abbreviations, see section 2.B.2.3.

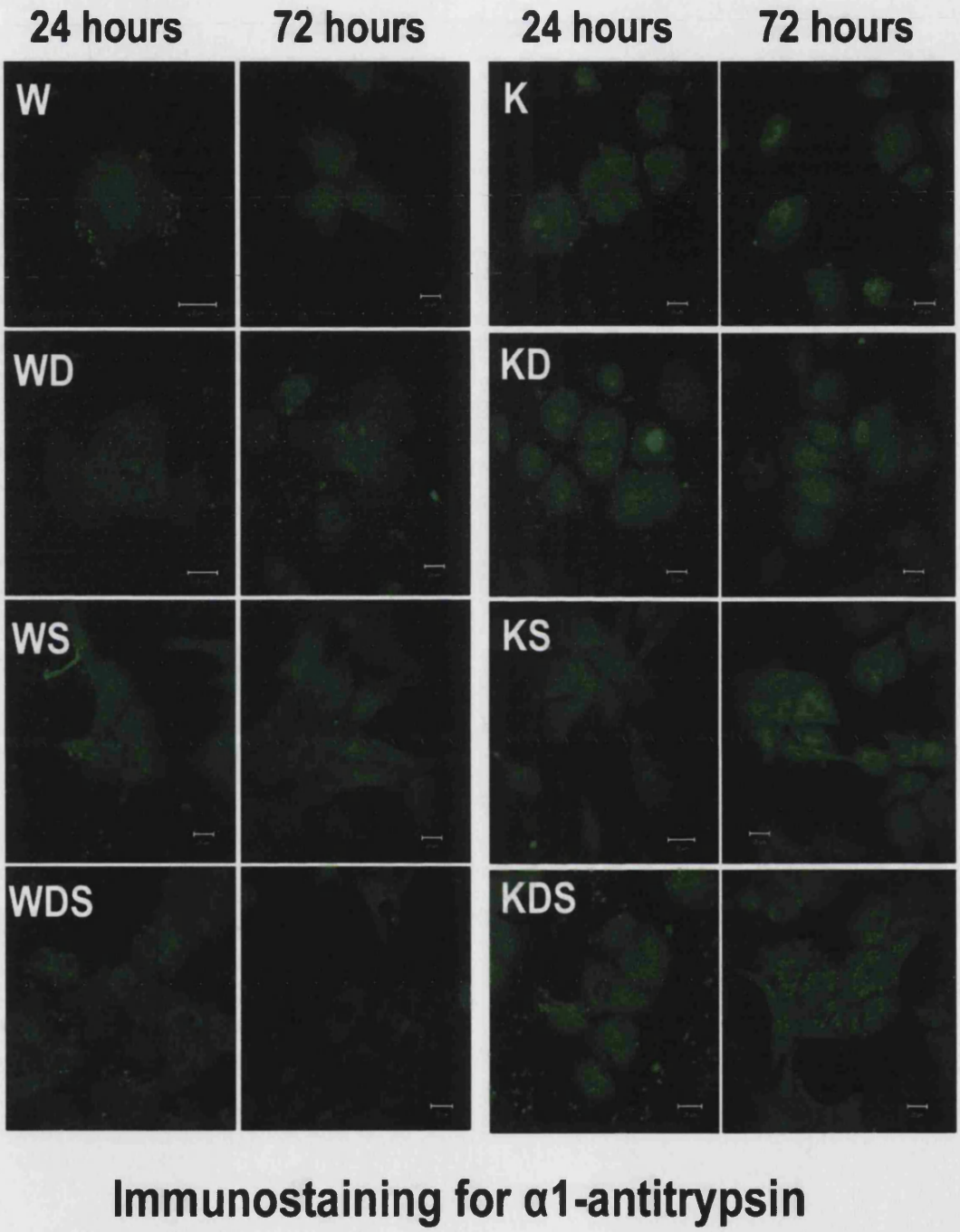


Figure 6.4 α1-antitrypsin expression in isolated rat hepatocytes cultured for 24 and 72 hours in different culture media. Scale bar = 20 μm.

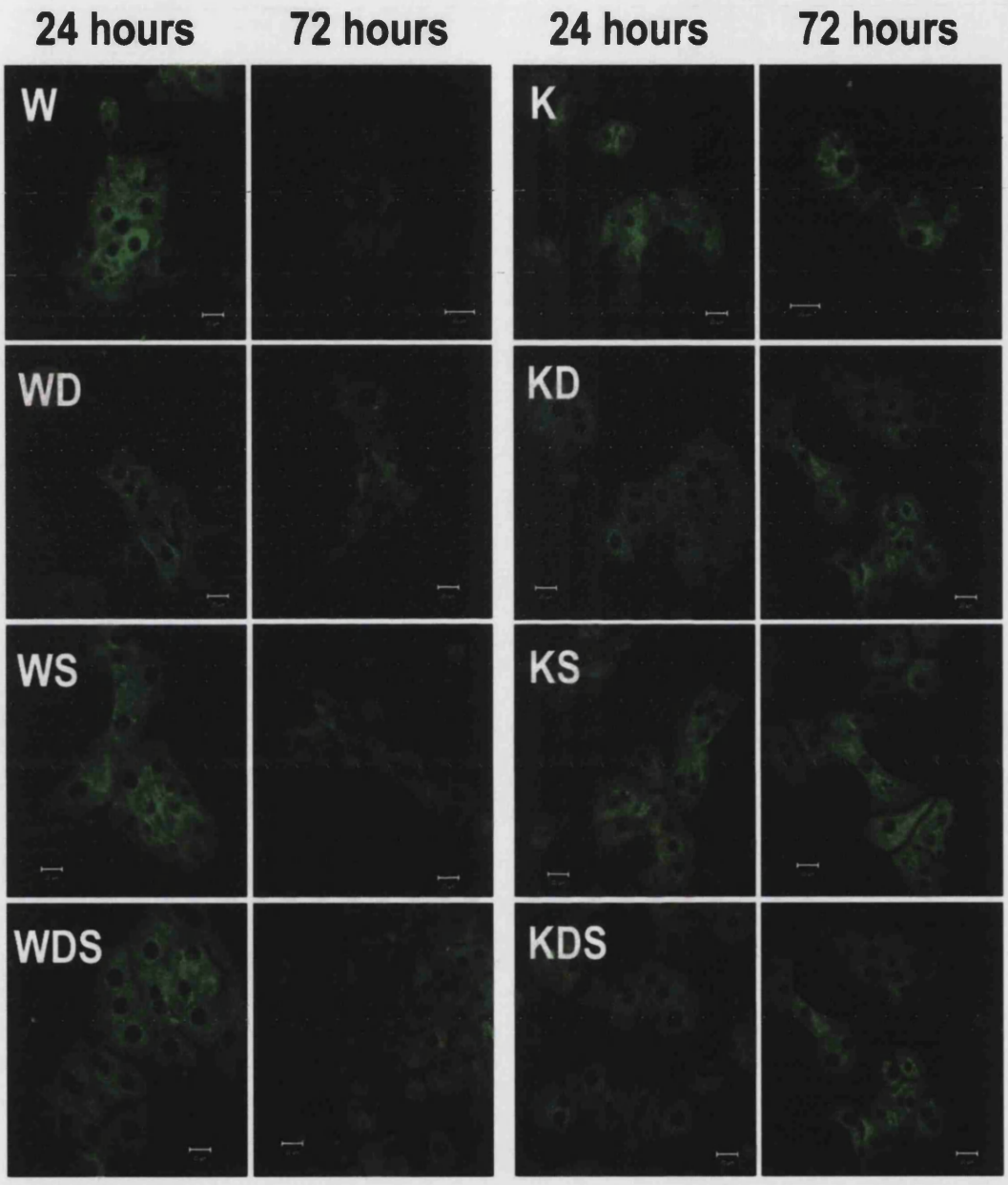


Figure 6.5 Haptoglobin expression in isolated rat hepatocytes cultured for 24 and 72 hours in different culture media. Scale bar = 20 μm.

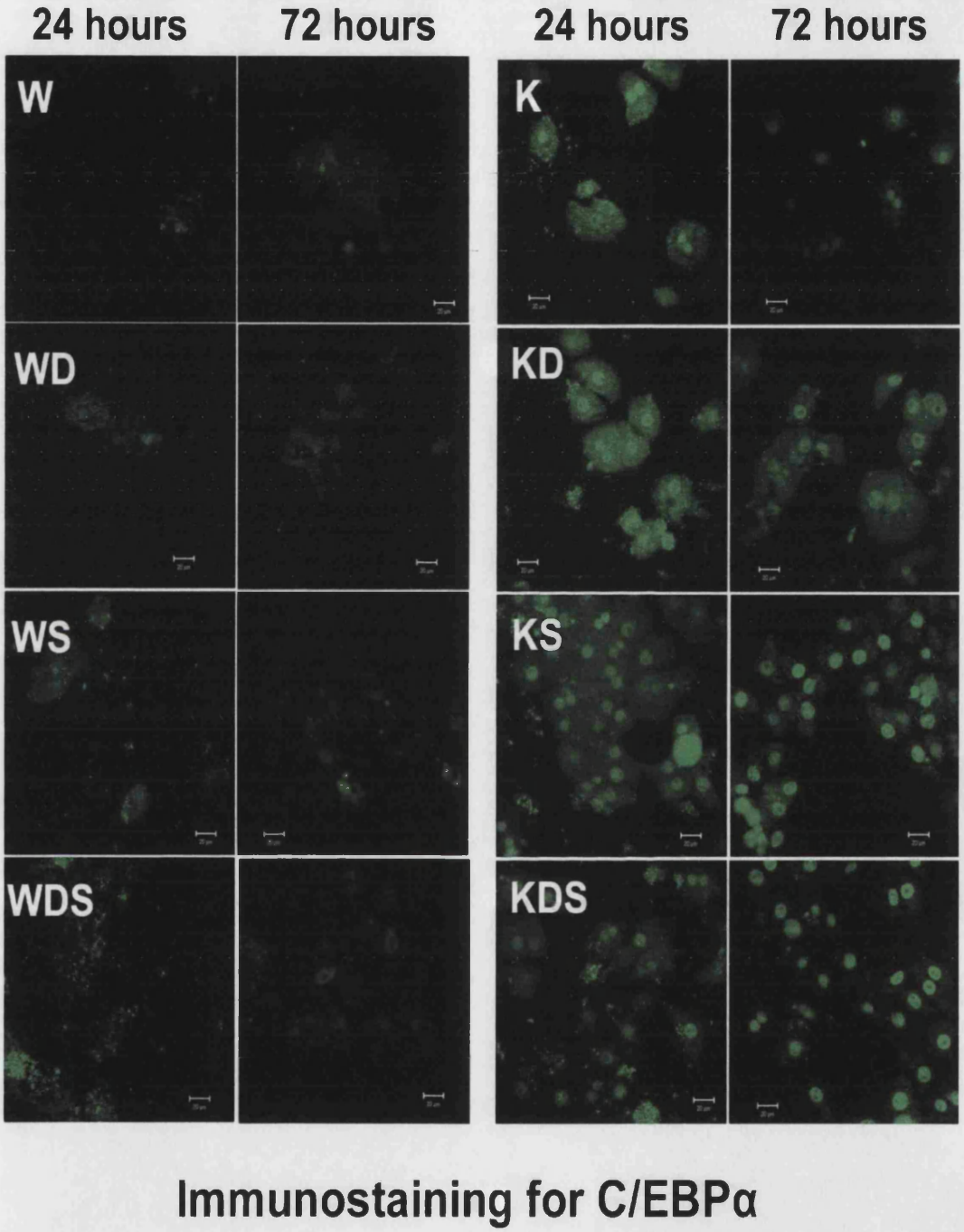


Figure 6.6 C/EBPα expression in isolated rat hepatocytes cultured for 24 and 72 hours in different culture media. Scale bar = 20 μm.

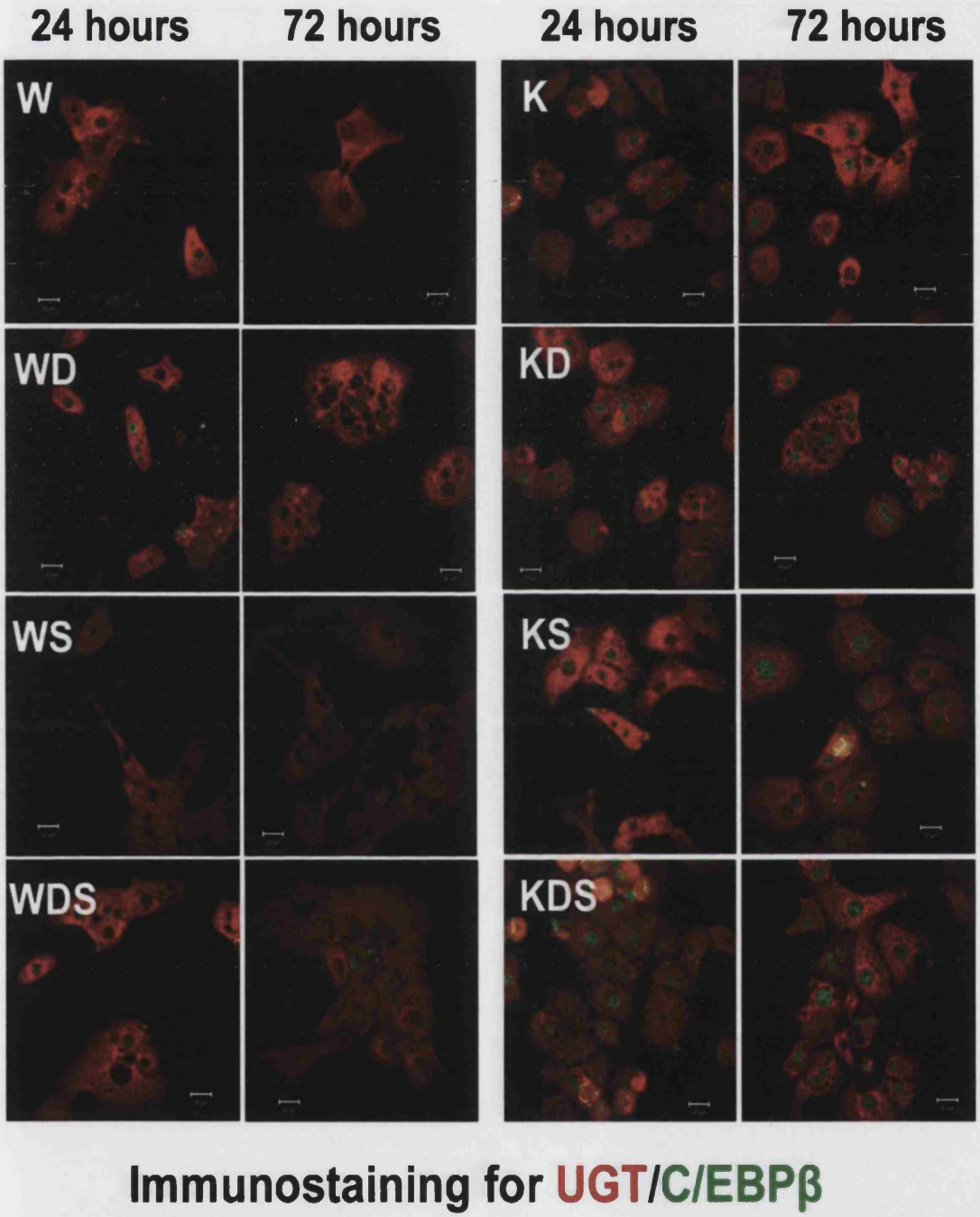


Figure 6.7 Expression of C/EBPβ (green) and UGT (red) in isolated rat hepatocytes cultured in different culture media for 24 and 72 hours. Scale bar = 20 μm.

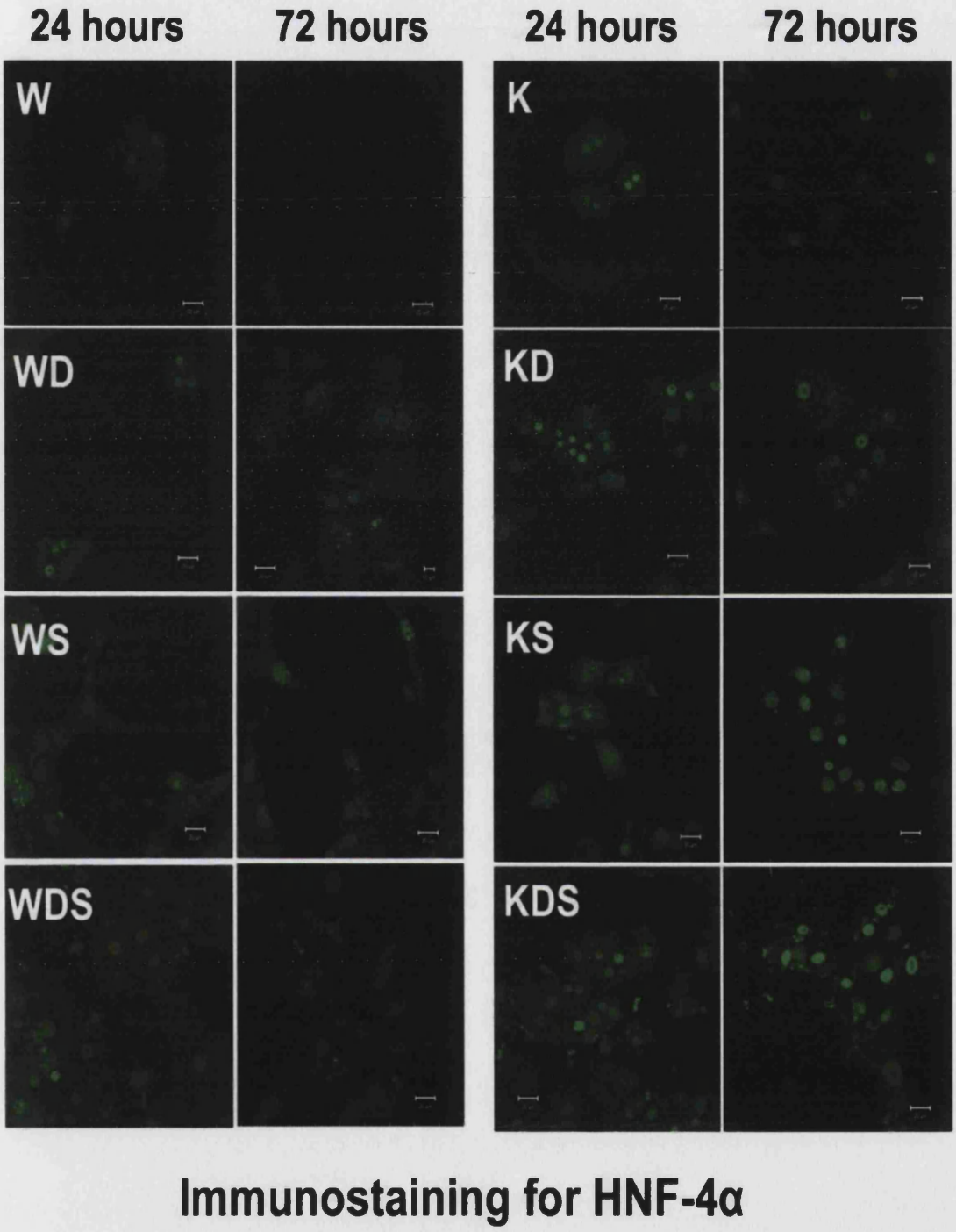


Figure 6.8 HNF4α expression in isolated rat hepatocytes cultured in different culture media for 24 and 72 hours. Scale bar = 20 μm.

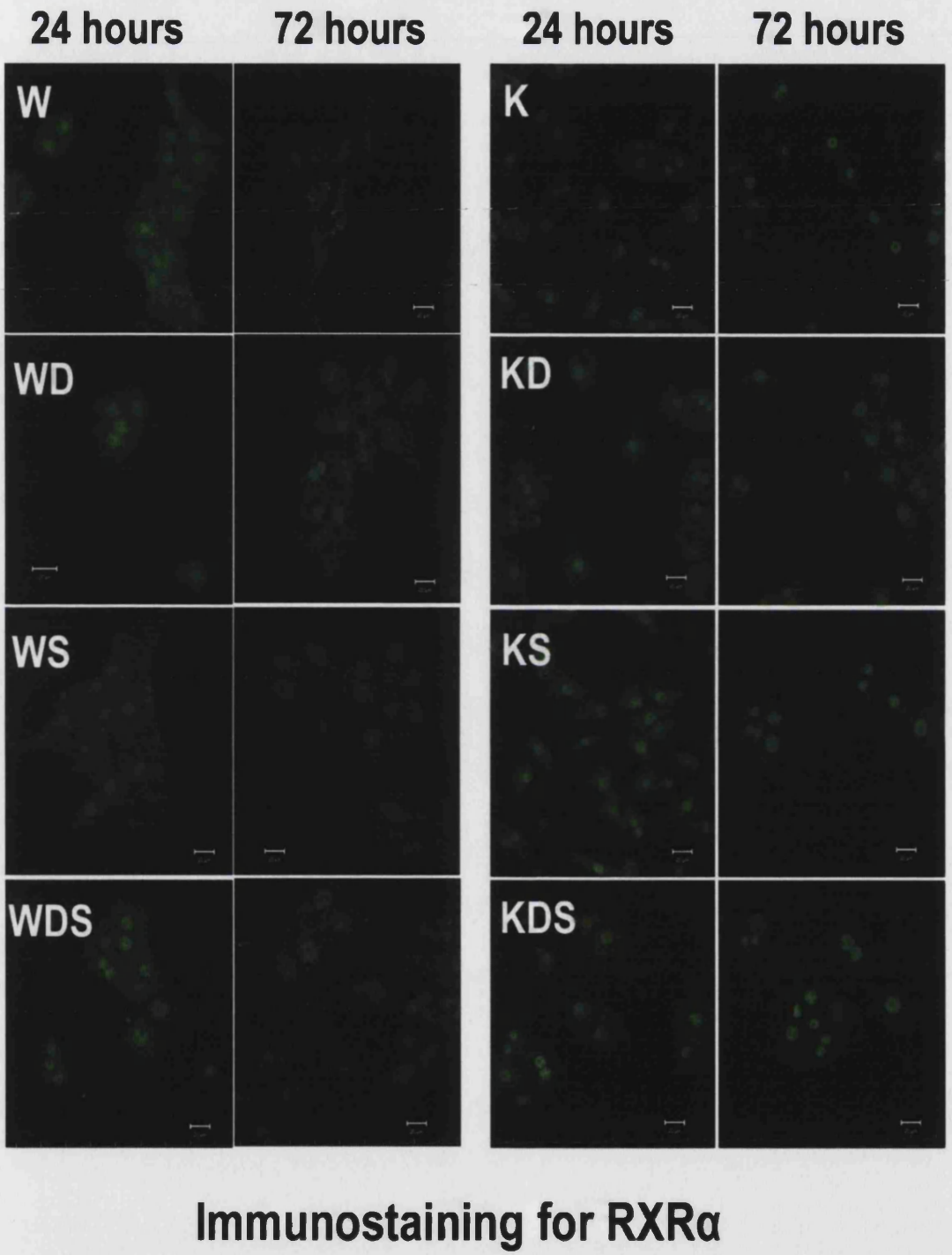


Figure 6.9 RXRα expression in isolated rat hepatocytes cultured in different culture media for 24 and 72 hours. Scale bar = 20 μm.

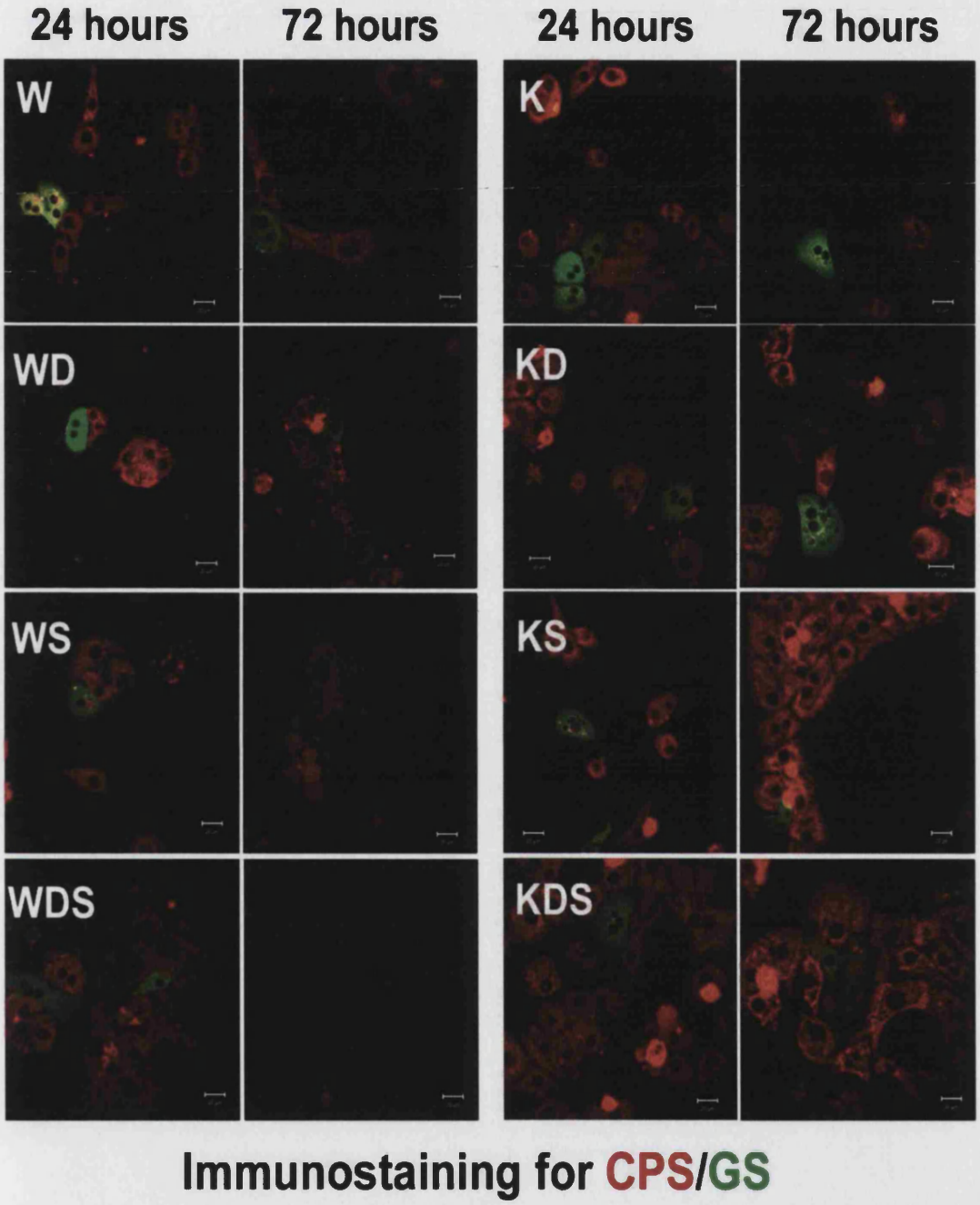


Figure 6.10 Glutamine synthetase (GS, green) and carbamoylphosphate synthetase (CPS, red) expression in isolated rat hepatocytes cultured in different culture media for 24 and 72 hours. Scale bar = 20 μm.

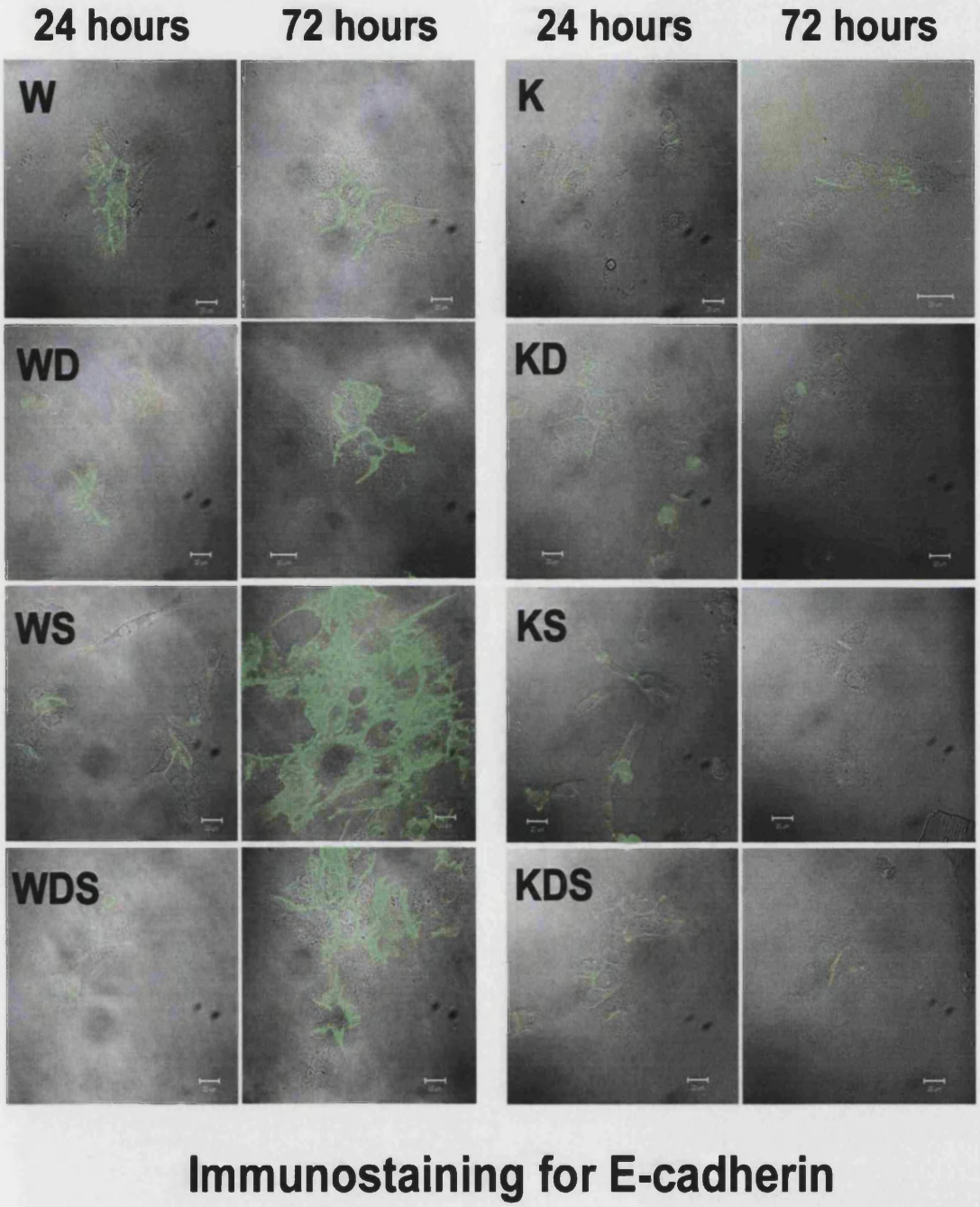


Figure 6.11 E-cadherin expression in isolated rat hepatocytes cultured in different culture media for 24 and 72 hours. Scale bar = 20 μm.

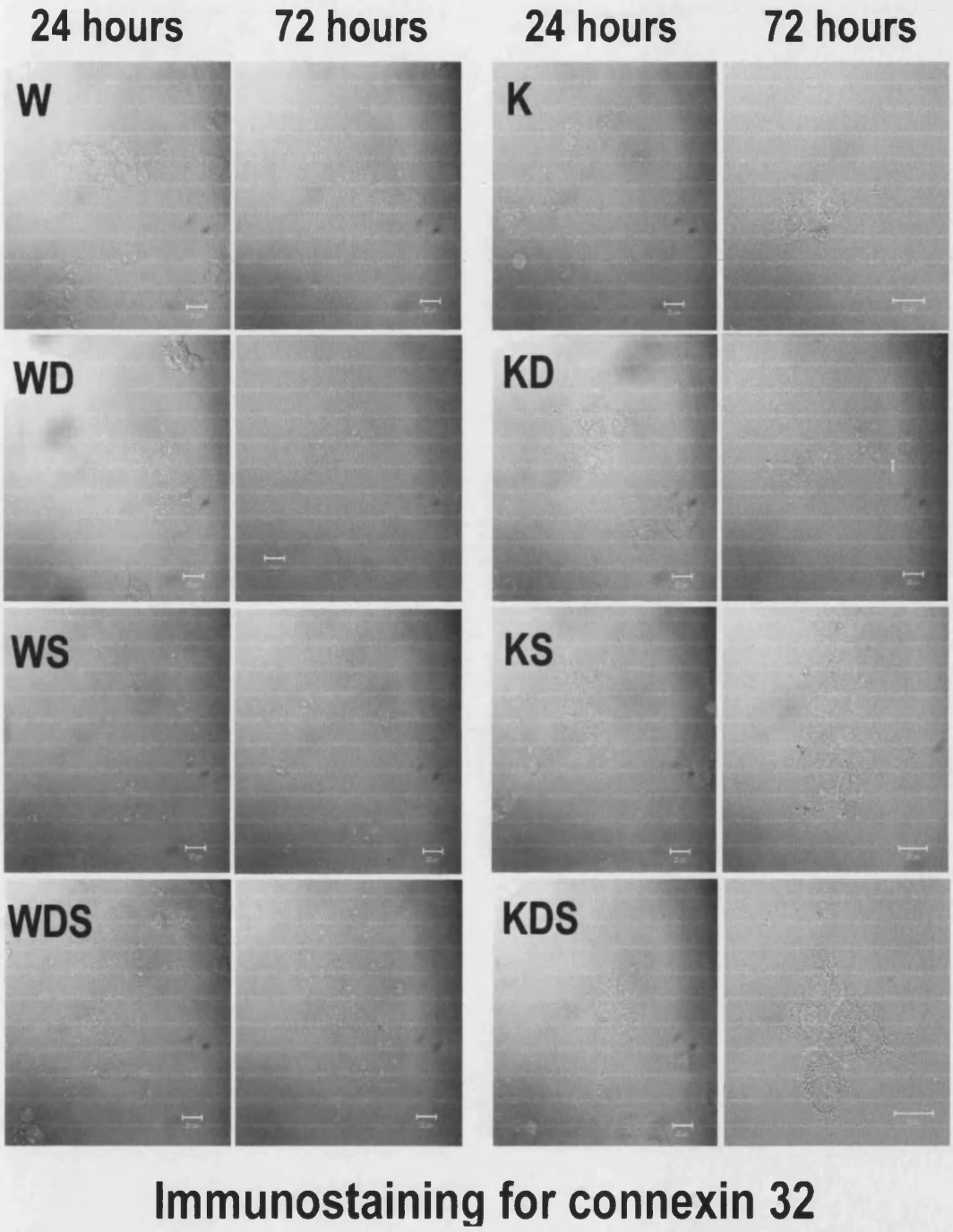


Figure 6.12 Connexin 32 expression in isolated rat hepatocytes cultured in different media for 24 and 72 hours. Scale bar = 20 μm.

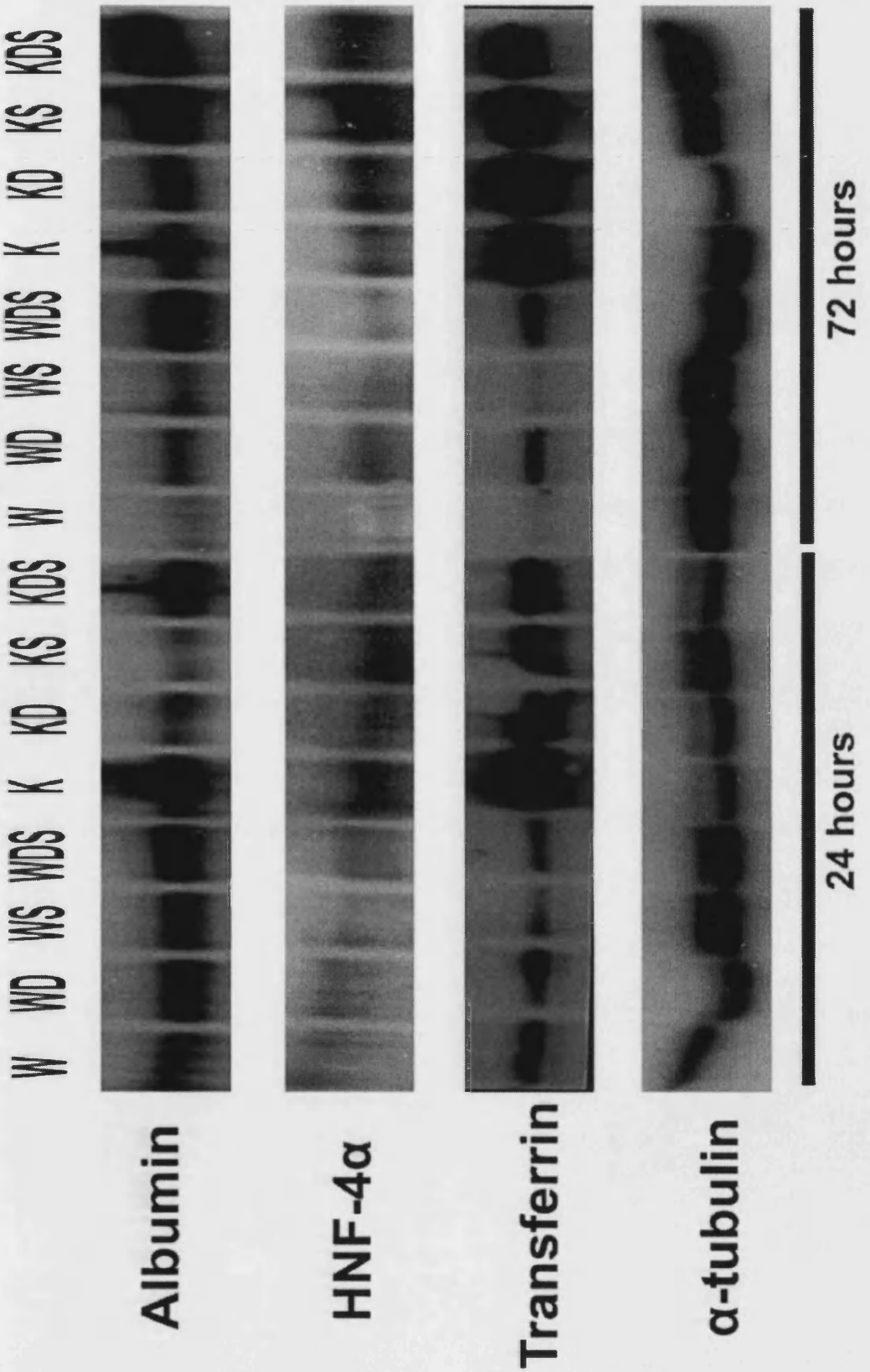

W
WD
WS
WDS
K
KD
KS
KDS
W
WD
WS
WDS
K
KD
KS
KDS
Albumin
HNF-4α
Transferrin
α-tubulin
24 hours
72 hours

Figure 6.13 Western blotting analysis for albumin, transferrin and HNF4α in 24-hour and 72-hour cultured rat hepatocytes. 10μg total protein was loaded per lane. α-tubulin was used as an internal loading control.

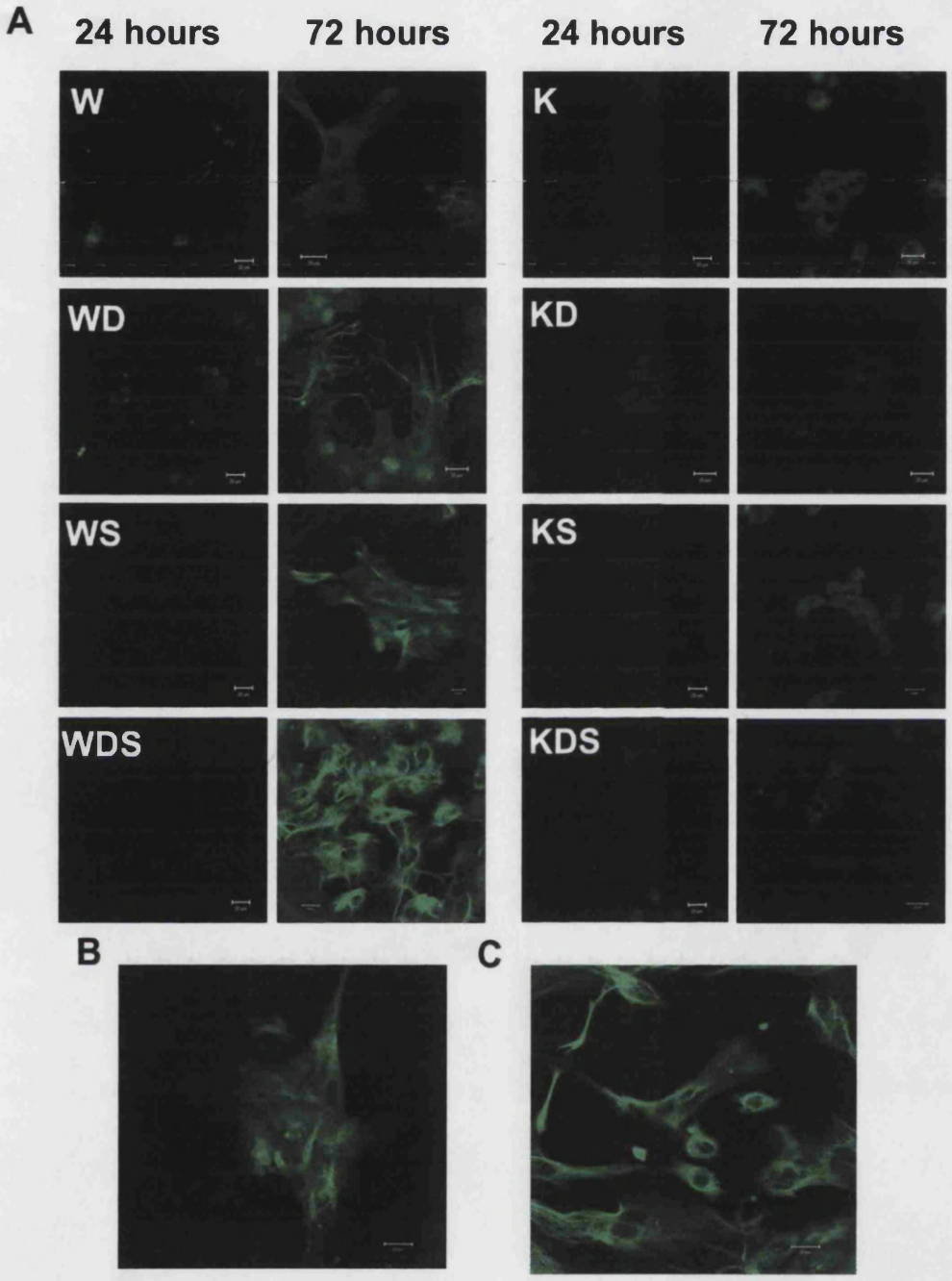


Figure 6.14 Vimentin expression in isolated rat hepatocytes cultured in different media for 24 and 72 hours (A). In 72-hour WS cultured rat hepatocytes. Scattered de-differentiated hepatocytes (B) and groups of vimentin positive cells (C) were observed. Scale bar = 20 μm.

6.B.2 Long-term preservation of differentiated rat hepatoctyes in KSFM culture medium

Although the results for three days were encouraging, it was important to determine whether KSFM media can maintain the hepatic phenotype for longer culture periods. In order to find out the optimal culture conditions, the survival of hepatocytes was examined under different culture conditions. Equal numbers of cells were seeded and cultured in six different KSFM culture conditions. The cell number, detected by DAPI staining of cell nuclei, at 24 hours of culture was set as 100% and the relative percentage of cell numbers at 1 week / 2 weeks / 3 weeks were determined (Figure 6.15). As cells cultured in KDS exhibited the best survival rates it was decided to use KDS for subsequent experiments.

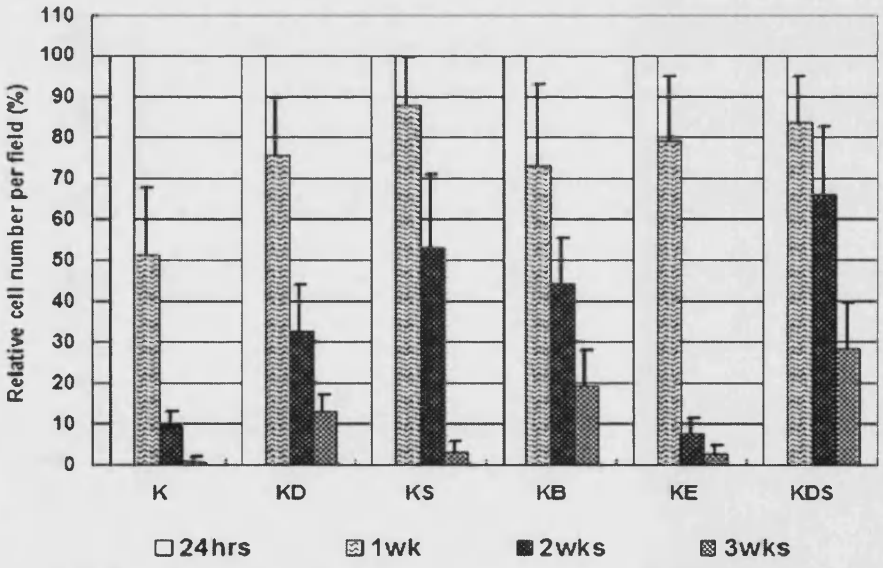


Figure 6.15 Time course for the percentage of surviving hepatocytes cultured in KSFM medium. The histogram describes the percentage of hepatocytes (based on DAPI-positive remaining from the 24 hour culture point). The data were obtained from at least 6 random fields for each culture and presented as mean ± S.D.. Abbreviations: K-Keratinocyte serum free medium; D-dexamethasone; S-supplemented with both EGF and BPE; B-bovine pituitary gland extract and E - recombinant human epidermal growth factor.

A number of liver specific markers were examined in KDS cultured rat hepatocytes using RT-PCR, Western blotting and immunostaining. For RT-PCR, the primers were designed for a number of enzymes including Phase I detoxification enzymes cytochrome P450s (Cyp2B12, Cyp3A1, Cyp7A1 and Cyp reductase), rate-limiting enzymes involved in amino acid metabolism such as tyrosine aminotransferase (TAT)(Yeoh et al., 1979) and tryptophan 2,3-dioxygenase (TdO)(Moore et al., 1986) and CPS. All these transcripts were highly expressed in KDS cultured rat hepatocytes up to 3 weeks and some (e.g. CPS, Cyp3A1, TAT and TdO) were still present at 4 weeks (Figure 6.16). Using Western blotting, hepatic proteins including albumin, transferrin, UGT, the acute phase protein haptoglobin and liver-enriched transcription factor HNF1α and HNF4α were detected and showed steady expression for up to 4 weeks (Figure 6.17). Furthermore, the expression of several liver-specific proteins such as apolipoprotein B (ApoB), transferrin, albumin, Cyp2E1 was also detected by immunostaining. The results indicated that these proteins could be maintained for 2 weeks (Figure 6.18), 3 weeks (Figure 6.19) and 4 weeks (Figure 6.20) under KDS culture conditions. In addition, C/EBPα, C/EBPβ, RXRα and HNF4α were maintained for at least 3 weeks.

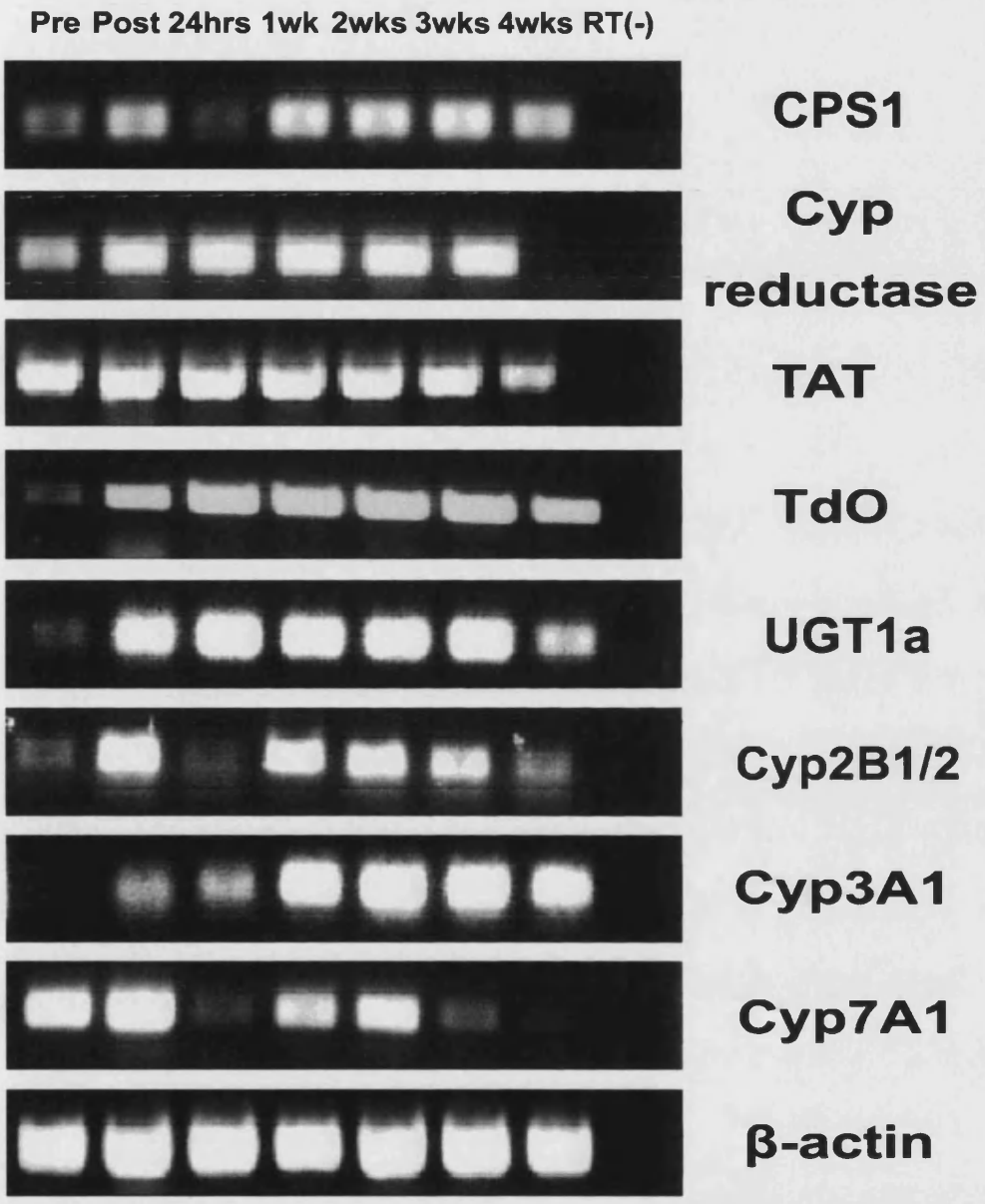


Figure 6.16 RT-PCR examination of hepatic mRNA expression in long-term KDS cultured rat hepatocytes. The expression of carbamoylphosphate synthetase I (CPS1), cytochrome reductase, tyrosine aminotransferase (TAT), tryptophan di-2,3-oxygenase (TdO), UDP-glucuronosyltransferase 1a (UGT1a) and three cytochrome P450 genes (Cyp2B12, Cyp3A1, Cyp7A1) are investigated. RT(-): no template control. Pre and Post represent the mRNAs extracted from normal liver or freshly isolated hepatocytes, respectively.

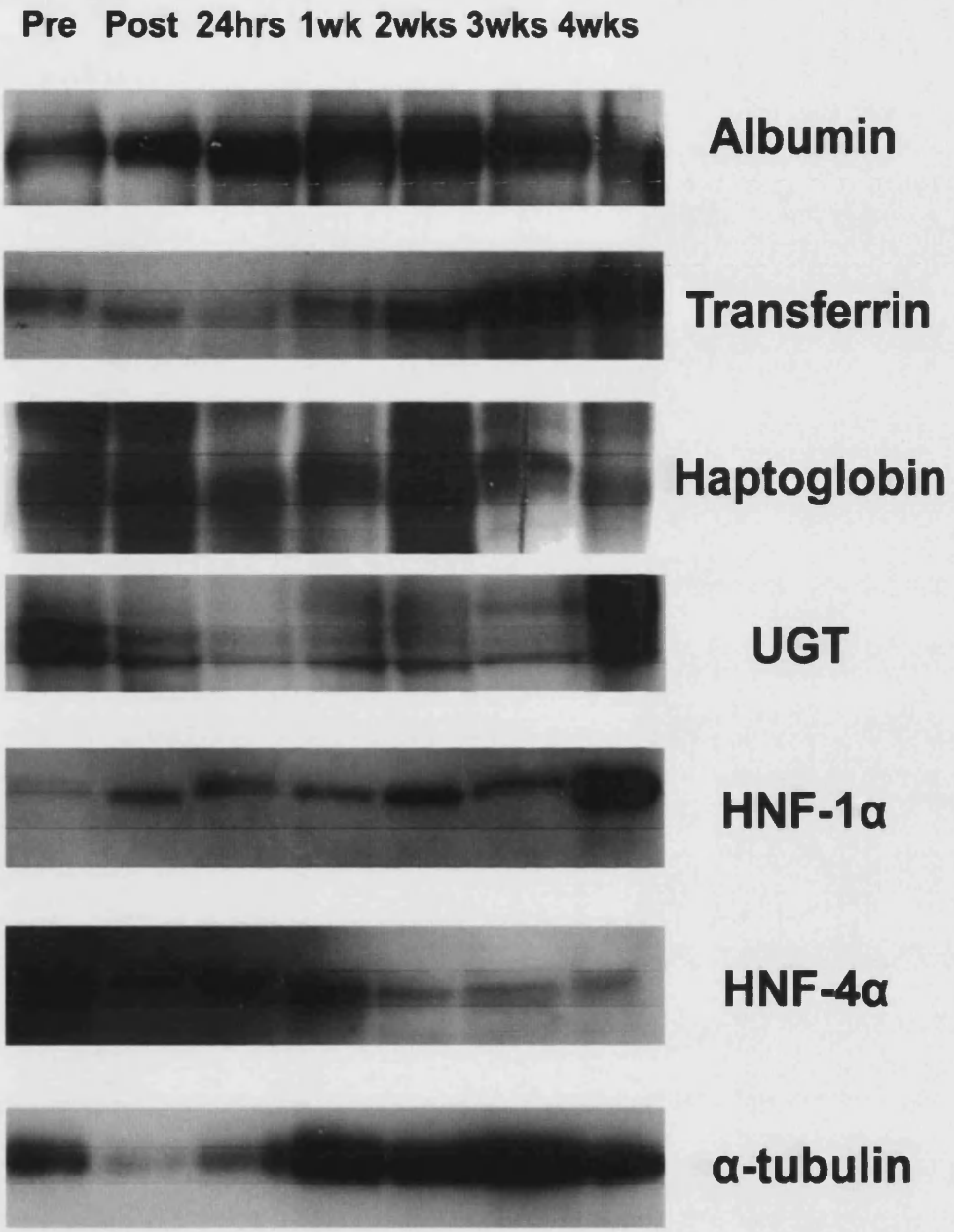


Figure 6.17 Time-course protein expression in KDS cultured rat hepatoctyes. Western blotting of albumin, transferrin, haptoglobin, UGT, HNF-1α and HNF-4α in hepatocytes cultured for different time periods (24hours to 4 weeks) in KDS cultured rat hepatoctyes. Pre and Post in (A) and (B) represent the proteins extracted from normal liver or freshly isolated hepatocytes, respectively.

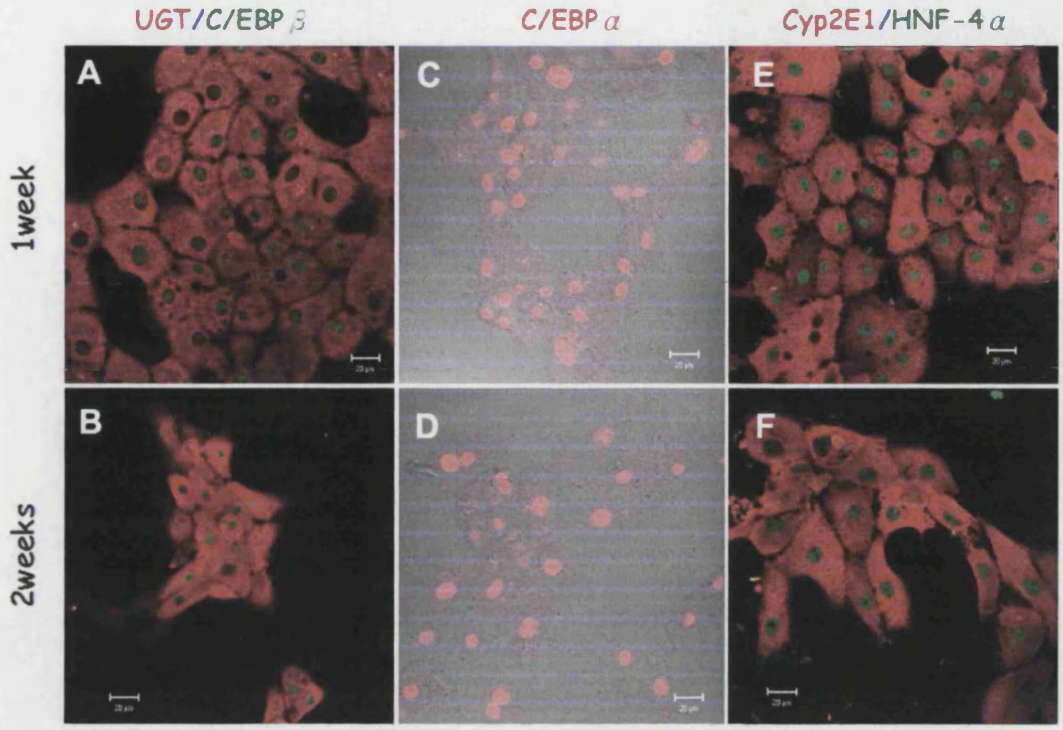


Figure 6.18 Protein expression in 1-week and 2-weeks KDS cultured rat hepatoctyes. Immunofluorescence staining for the expression of C/EBPβ (green) and UGT (red) (A,B), C/EBPα (C,D) and HNF4α (green) and Cyp2E1 (red) in 1-week and 2-week KDS cultured rat hepatocytes. Scale bar = 20 μm.

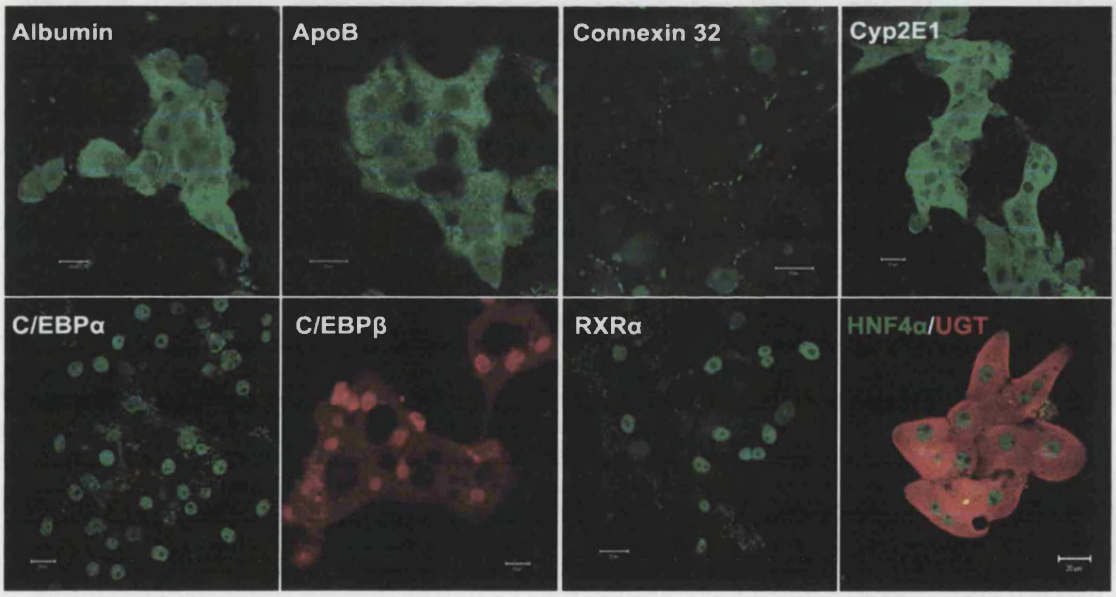


Figure 6.19 Protein expression in 3-weeks KDS cultured rat hepatoctyes. Immunofluorescence staining for albumin, ApoB, Connexin 32, Cyp2E1, UGT, C/EBPα, C/EBPβ, HNF4α and RXR in rat hepatocytes cultured for 3 weeks in KDS. Scale bar = 20 μm.

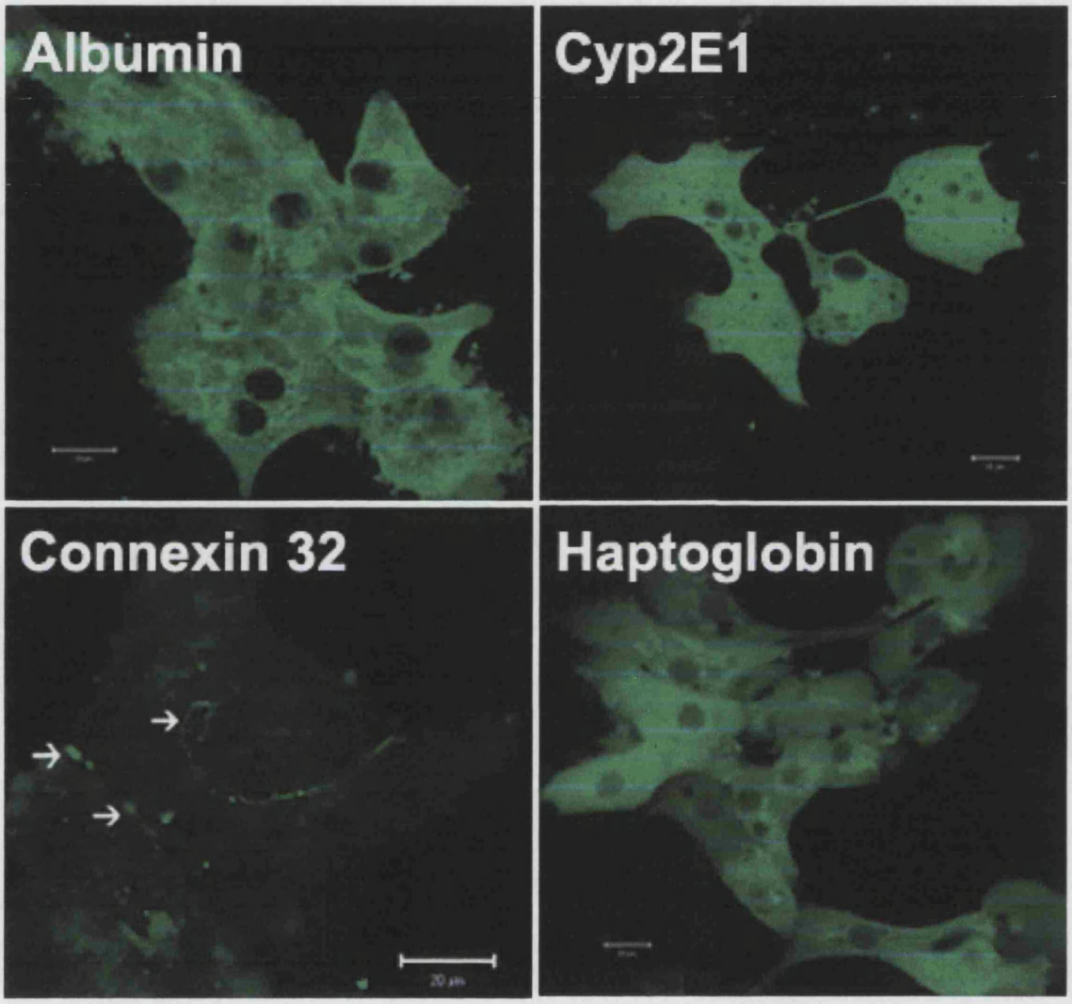


Figure 6.20 Protein expression in 4-weeks KDS cultured rat hepatoctyes. Immunofluorescence staining for albumin, Connexin 32 (indicated by arrows), Cyp2E1 and haptoglobin rat hepatocytes cultured for 4 weeks in KDS media. Scale bar = 20 μm.

6.B.3 Functional analysis of KSFM cultured rat hepatoctyes

6.B.3.1 Lipid and glycogen storage in KDS cultured rat hepatocytes

To test whether hepatocytes cultured under KDS conditions maintained their functional capacity, their potential to store lipid and to oxidise glucose into glycogen was examined. Oil red O is one of the Sudan dyes used for the detection of general lipids(Bayliss and Adams, 1972). Oil red O was used to stain the lipid droplets in hepatocytes. By this means, the lipids were seen to be present in the hepatocytes cultured with KDS up to 4 weeks after isolation (Fig 6.21). The examination of glycogen in the KDS cultured hepatocytes was performed using Periodic acid- Schiff (PAS) staining (Lazaro et al., 2003). The results showed that glycogen storage could also be maintained in the hepatocytes cultured with KDS for up to 4 weeks after the induction of the culture medium containing 25mM glucose (Figure 6.22).

6.B.3.2 Active urea cycle in KDS cultured rat hepatocytes

The urea cycle is an essential metabolic process for the removal of ammonia formed by the breakdown of many nitrogenous compounds in liver (Morris, 2002). In brief, arginine from the diet or from protein breakdown is cleaved by the cytosolic enzyme arginase, generating urea and ornithine. Ornithine in the cytosol is transported to the mitochondrial matrix, where ornithine transcarbamoylase catalyses the transformation of ornithine to citrulline. The citrulline is then transported to the cytoplasm, and converted to argininosuccinate by argininosuccinate synthetase,. Then arginine and fumarate are produced from argininosuccinate by the cytosolic

enzyme argininosuccinate lyase (also called argininosuccinase) and, in the final step of the cycle, arginase cleaves urea from arginine, regenerating cytosolic ornithine, which can be transported to the mitochondrial matrix for another round of urea synthesis.

The activity of ureagenesis in the KDS cultured rat hepatocytes was examined by two separate assays, the urea assay and the arginase assay. Assays were performed after 24 hours, 1 week, 2 weeks, 3 weeks and 4 weeks of culture using either the detection of secreted urea (urea assay), or by measurement of arginase activity (arginase assay). Arginase activity was detected at each time point while the level of urea production remained stable (between 1600 µg and 2100 µg urea per dish of cells) (Figure 6.23). For the urea assay, the culture medium was changed 24 hours prior to the assay and the results suggested that secreted urea was slightly reduced after 4 weeks culture (Figure 6.24). Although it is impossible to interpret these results with *in vivo* data, stabilisation of urea cycle activity suggested that the urea cycle is active for more than 2 weeks under KDS culture conditions.

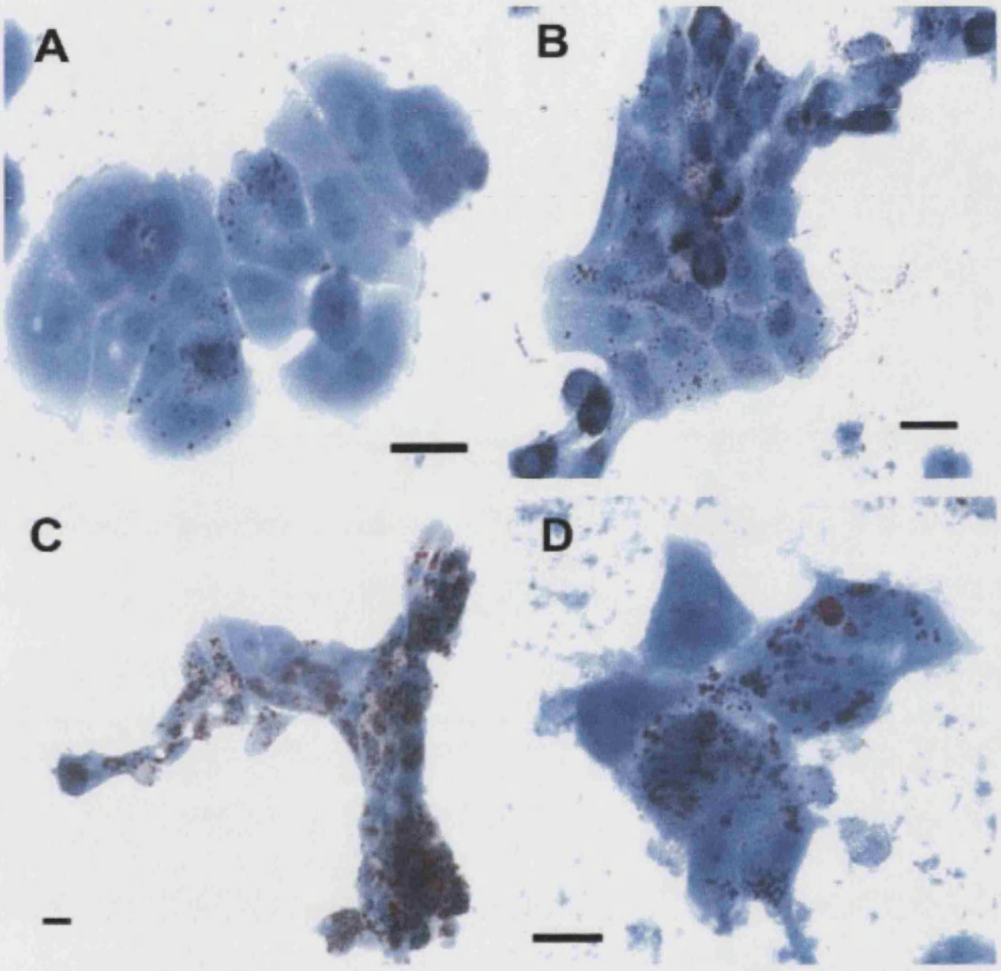


Figure 6.21 Lipid storage in rat hepatocytes cultured in KDS medium. Lipid droplets were stained in rat hepatocytes by oil red O staining at (A) 24-hours (B) 1-week (C) 2-weeks (D) 4-weeks of culture. Scale bar = 20 μm.

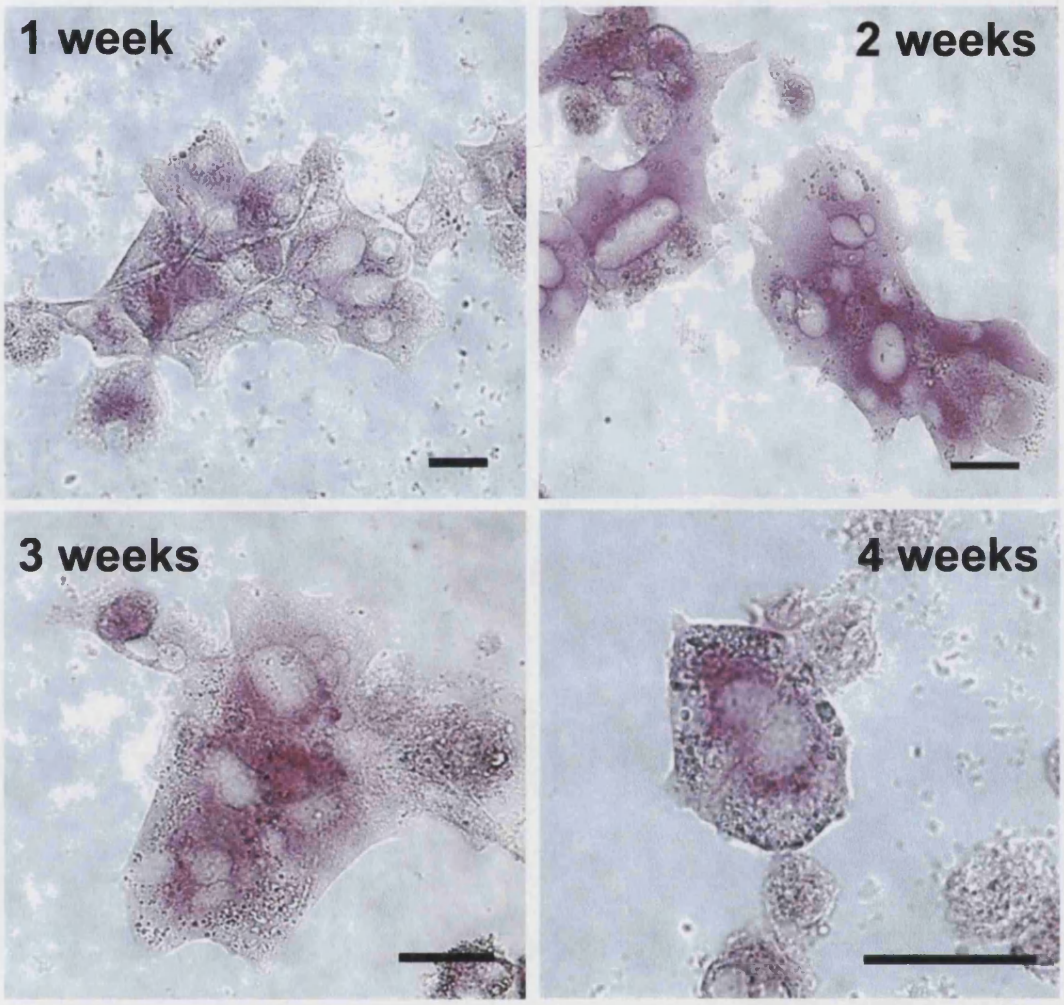


Figure 6.22 Glycogen storage in rat hepatocytes cultured in KDS medium using the PAS assay. Glycogen was stained in rat hepatocytes at (A) 1-week (B) 2-week (C) 3-week (D) 4-weeks of culture. Scale bar = 20 μm.

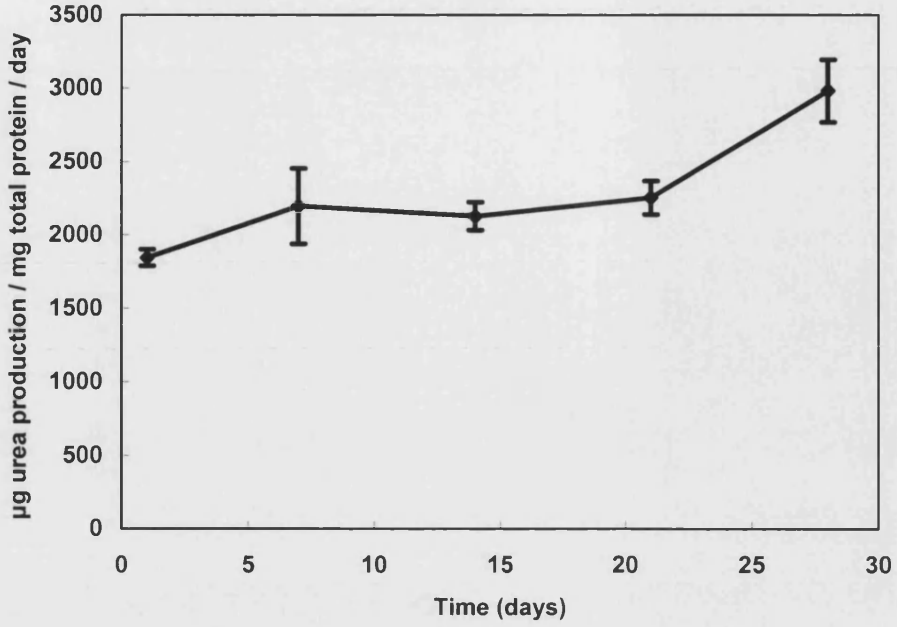


Figure 6.23 Arginase activities in cultured rat hepatocytes. The activity of arginase was determined using the procedure described in section 2.B.10.2. The data are represented as mean ± S.D. and from at least 4 individual dishes.

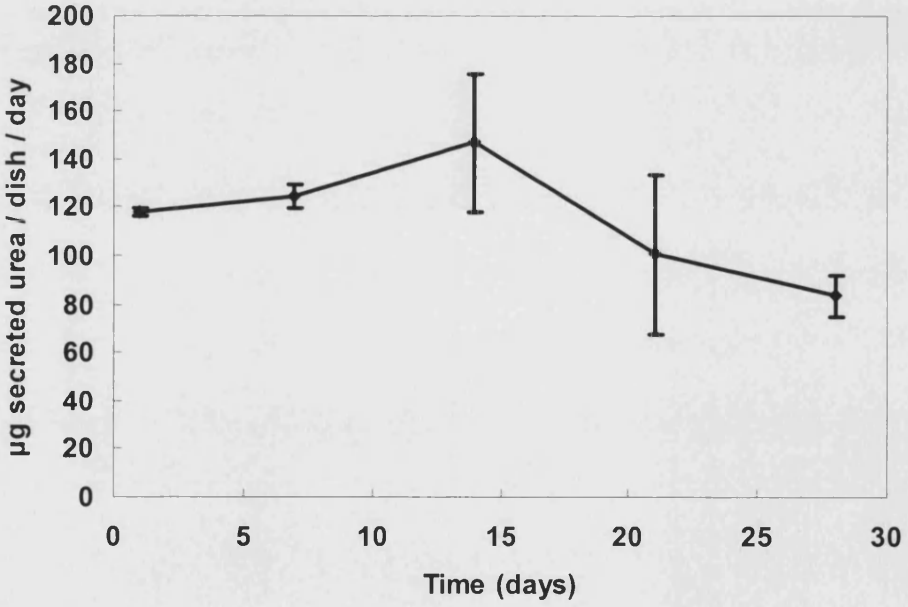


Figure 6.24 Urea secretion cultured rat hepatocytes. Rat hepatocytes were cultured for different time points (24-hours to 4-weeks) in KDS media. The media were collected and the urea concentration determined using the procedure described in section 2.B.10.1. The data are represented as mean ± S.D. and from at least 4 individual dishes.

6.B.4 Hepatic metabolising enzymes are induced by phenobarbital treatment in KDS cultured hepatocytes

"Xenobiotic" is the term used to describe a foreign particle or molecule that is potentially dangerous or toxic (Waxman et al., 1990). Phenobarbital, one common xenobiotic, is used as an inducer of Phase I and II metabolising enzymes in liver (Waxman et al., 1990). Following treatment by phenobarbital, the transcription of phase I (cytochrome P450s) and phase II (UGT) detoxification enzymes is activated. It is through the binding of CAR-RXR (CAR, constitutive active receptor; RXR, 9-*cis*-retinoic acid receptor) heterodimers with the cis-acting element (DR4 motifs, such as NR1 and NR2 domain on the enhancer of *Cyp2b10* gene) of the genes (Kakizaki et al., 2003; Swales and Negishi, 2004). To examine the effect of phenobarbital on Phase I and II metabolising enzymes in KDS cultured rat hepatocytes, the expression of *Cyp2B12*, *Cyp3A1*, *Cyp7A1* and *UGT* were enhanced by real-time RT-PCR analysis. Rat hepatocytes were cultured for 1 week in KDS and then treated with and without phenobarbital for 3, 5 or 7 days (i.e. in total for 10, 12 or 14 days). All the Phase I and Phase II genes examined were upregulated 2-10-fold in phenobarbital-treated cells compared with control cells (Figure 6.25). For longer-term effects, the KDS cultured hepatoctyes were incubated for 1,2,3 and 4 weeks and then treated with 10μM phenobarbital for a further 3 days. KDS cultured hepatocytes respond to phenobarbital treatment. The UGT and cytochrome P450s (Cyp2B12, Cyp3A1 and Cyp7A1) (Figure 6.26) were upregulated for at least 2 weeks. However, the response to phenobarbital after 3 and 4-weeks was lower compared to treatment after 2 weeks. The reduced response after 3 or 4 weeks might be due to lower cell viability. Approximately 50% of cells detached between 2 and 3 weeks of culture

(Figure 6.15). The results suggest that KDS cultured rat hepatocytes are able to respond appropriately to phenobarbital and hence may prove a useful system for drug screening *in vitro*.

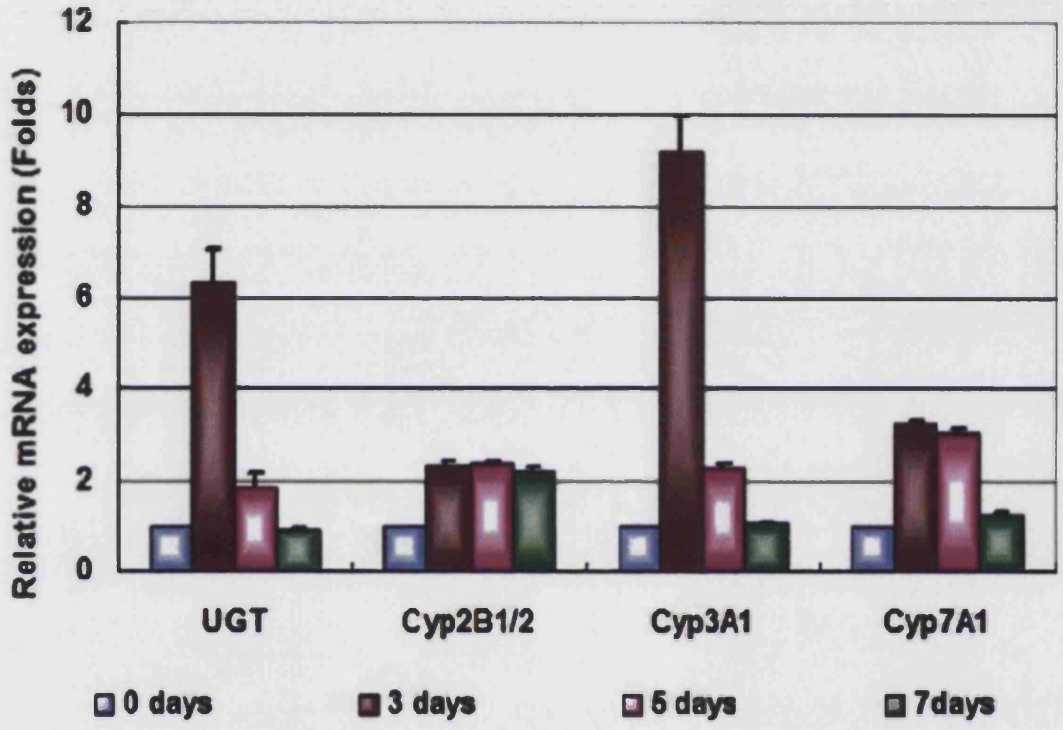


Figure 6.25 Phenobarbital-induced expression of liver metabolising enzymes. Hepatocytes were cultured with KDS for 7 days and then the medium was changed to KDS supplemented with and without 10 μM phenobarbital for another 3, 5 and 7 days. The expression of UGT, Cyp2B12, Cyp3A1 and Cyp7A1 was performed by real-time RT-PCR. The results were displayed as the ratio of (Conc. $_{[\text{target gene (treated)}]}$ / Conc. $_{[\text{reference gene (treated)}]}$):(Conc. $_{[\text{target gene (untreated)}]}$ / Conc. $_{[\text{reference gene (untreated)}]}$). The calibrator is the mRNA from adult rat liver.

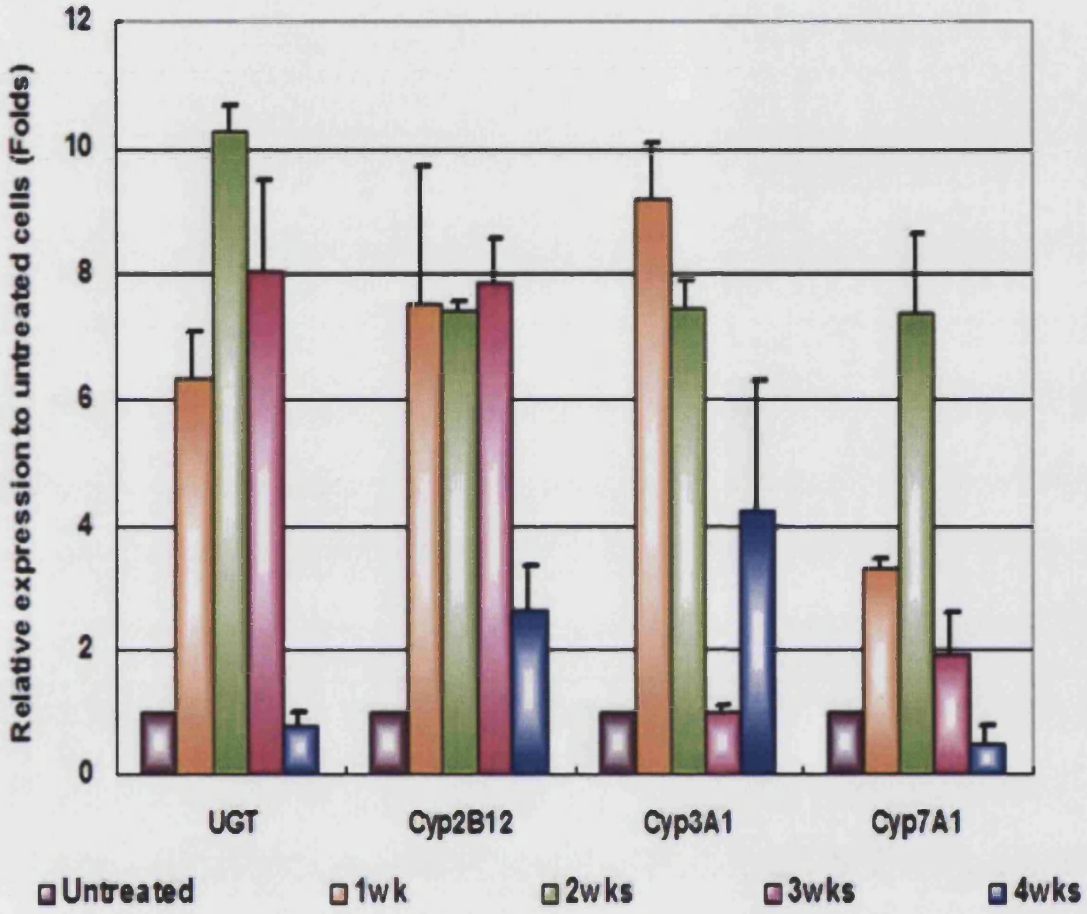


Figure 6.26 Induction of Phase I or II metabolising enzymes in response to phenobarbital treatment of long-term KDS cultured rat hepatocytes. The experimental procedure and the result are the same as described in Figure legends of Figure 6.25.

6.B.5 Effect of EGF and BPE on hepatic gene expression in transdifferentiated hepatocytes

It was then examined the effect of supplements (rhEGF+BPE) on the hepatic phenotype in transdifferentiated hepatocytes (TD hepatocytes) derived from the pancreatic cell line AR42J-B13 (B13). Hepatocytes are induced from B13 cells following treatment with 1μM dexamethasone(Shen et al., 2000). TD hepatocytes express a number of hepatic properties similar to normal hepatocytes(Burke et al., 2006; Kurash et al., 2004; Tosh et al., 2002a; Tosh et al., 2002b). It is interesting to know whether TD hepatocytes also respond to the supplements used for KSFM culture. TD hepatocytes were treated as shown in Figure 6.27. The expression of UGT and several liver mRNAs including serum protein albumin and α1-antitrypsin, cytochrome P450 enzyme Cyp7A1 and hepatic enzyme TAT was analysed using immunostaining and real-time RT-PCR. In cells cultured with supplements (either in the presence or absence of dexamethasone after 7 days) the number of UGT positive cells and UGT expression in TD hepatoctyes was enhanced (Figure 6.28). Furthermore, the hepatic genes albumin, α1-antitrypsin, Cyp7A1, HNF4α and TAT were upregulated in the cells incubated with supplements (Figure 6.29). These data confirmed that addition of EGF and BPE can enhance the hepatic phenotype in both cultured rat hepatocytes and in TD hepatocytes.

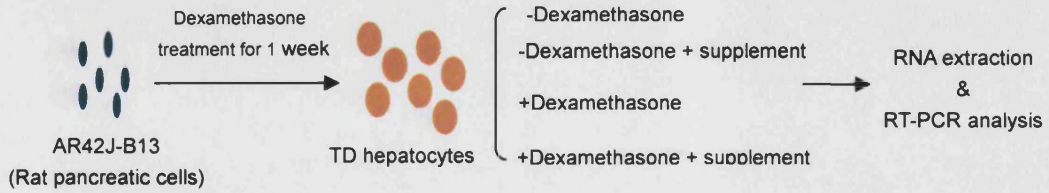


Figure 6.27

Figure 6.27

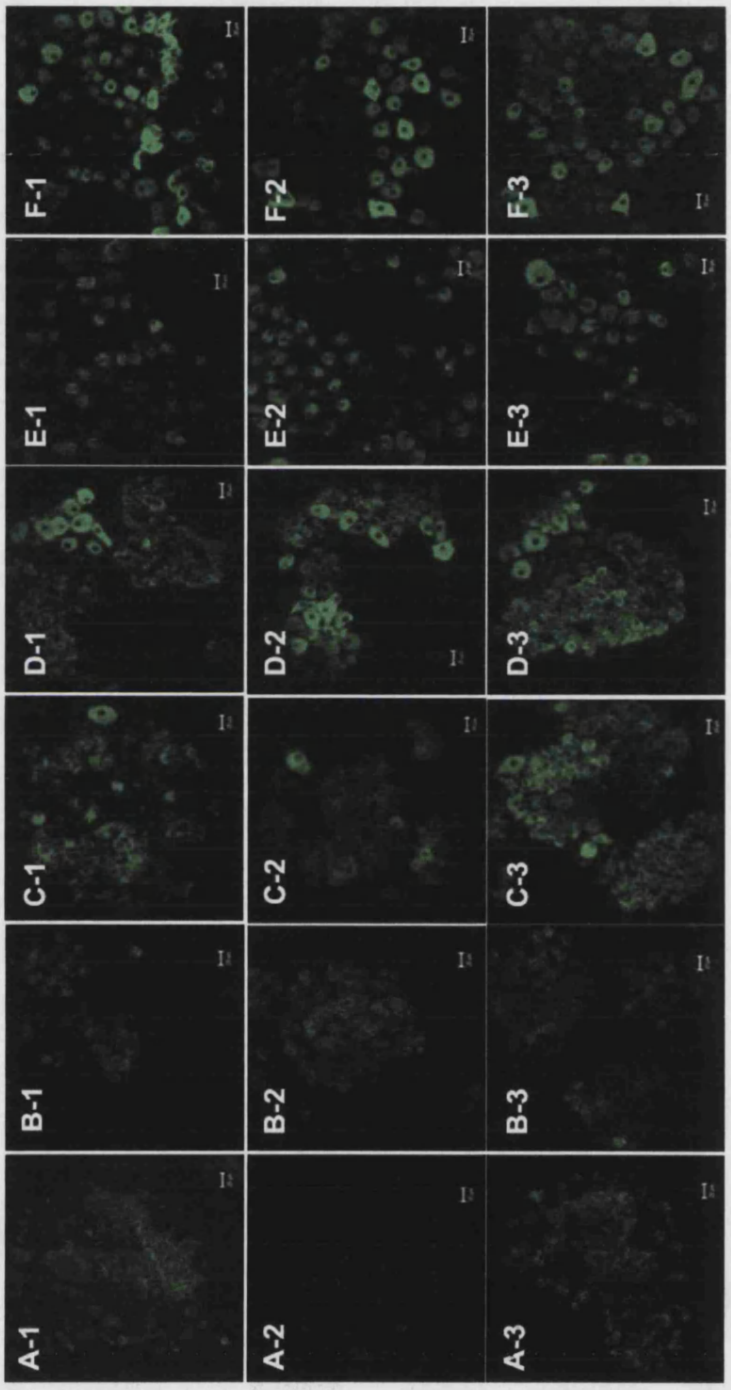

A-1
B-1
C-1
D-1
E-1
F-1
A-2
B-2
C-2
D-2
E-2
F-2
A-3
B-3
C-3
D-3
E-3
F-3
Dexamethasone --- --- 1-7 days 14 days 1-7 days 14 days
Supplements (rhEGF+BPE) --- 14 days --- --- 7-14 days 7-14 days

Figure 6.27 Scheme for investigating the effect of KSFM on tansdifferentiated hepatocytes induced from pancreatic AR42J-B13 cells.

Figure 6.28 Immunostaining of UGT expression in transdifferentiated hepatocytes. Hepatocytes were induced by culturing AR42J-B13 cells for 14-days with or without 1μM dexamethasone or 5ng/ml rhEGF and 50μg/ml PGE. Three typical fields are shown for each culture condition. Scale bar = 20μm.

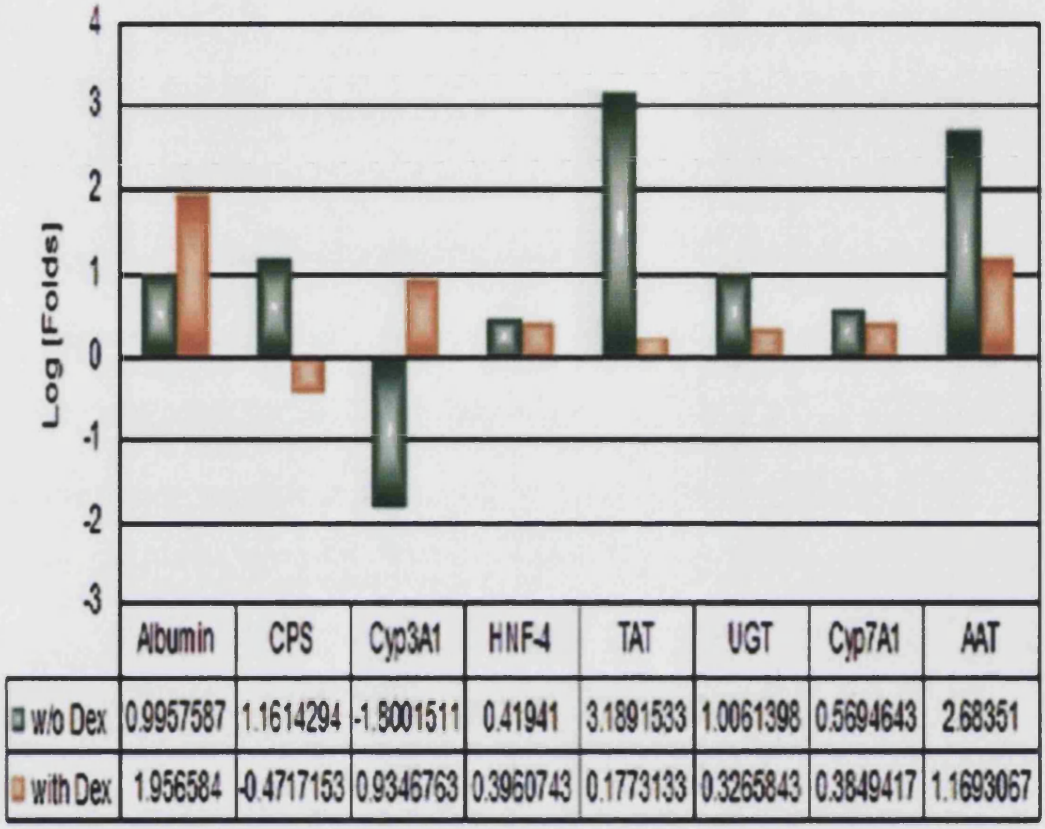


Figure 6.29 The effect of rhEGF and BPE on hepatic mRNA expression in cultured transdifferentiated hepatocytes. The expression of α1-antitrypsin, AAT; Carbamoylphosphate synthetase, CPS; tyrosine aminotransferase, TAT and UDP-glucuronosyltransferase, UGT; was performed by RT-PCR analysis. w/o Dex: culture in medium without containing dexamethasone; with Dex: medium containing dexamethasone. The numbers presented is log value of ratio of $mRNA_{\text{cell cultured with rhEGF and BPE}}$ / $mRNA_{\text{cell cultured without rhEGF and BPE}}$.

6.C Conversion of hepatocytes to β-like cells

Using rat hepatocytes maintained in KSFM media we sought to investigate whether it may be possible to induce the transdifferentiation to pancreatic cell types. Adenoviral infection was chosen since it more efficient compared to other transfection systems e.g. transfection (for further details see section 3.G). Tests of the infectious titre of adenoviral vector on the rat hepatocytes were first performed. Different titres of adenovirus containing expressing CMV-EGFP were infected into KDS cultured rat hepatocytes and then the cells were cultured for 2, 4 and 6 days. The infection efficiency was determined by the percentage of GFP positive cells at each time point. There is a balance between maintaining healthy cells (Figure 6.30) while at the same time ensuring the best infection efficiency (Figure 6.31).

Based on the preliminary results, it can be concluded that a multiplicity of infection (MOI) of 10-20 is optimal for virus infection in rat hepatocytes. Using an MOI of 20 for the TTR-Xlhbox8Vp16 adenovirus, insulin-expressing cells, either with or without co-expression of the hepatic marker albumin, were detected on the 5th day after infection (Figure 6.32). However, the transdifferentiation efficiency was still very low and it suggested that Xlhbox8Vp16 alone might not be sufficient to convert all hepatocytes into pancreatic-like cells.

Based on the notion of regulatory network for pancreas development (see Figure 1.1) (Edlund, 1998; Wilson et al., 2003), we hypothesised that perhaps the combination of several pancreatic specific transcription factors can increase the transdifferentiation efficiency (and in particular for β-cell generation). The different transcription factors (with a total viral titre of 20 MOI) were introduced onto KDS cultured rat hepatocytes and the gene expression for pancreatic β cells was then

analysed (Figure 6.33). These transcription factors included: Xlhbox8Vp16, Ngn3, Nkx2.2 and Nkx6.1. Following infection, the mRNAs were extracted and RT-PCR analysis performed for the expression of several β-specific genes including chromogranin A (ChA), GLP1 receptor (GLP-1r), islet amyloid polypeptide (IAPP), insulin, potassium channel subunit (Kir6.2), sulphonylurea receptor (SUR) and rat Pdx1. The results showed some reprogramming from a hepatic towards a pancreatic β cell phenotype after 5 days. β-cell specific genes were expressed to a higher level in rat hepatocytes infected with 3 or 4 transcription factors compared with those infected with only 1 or 2 transcription factors (Figure 6.34B). It is also worth noting that after 2 days of adenovirus infection, no insulin is detected but by 5 days the hormone is expressed. This suggests that the timing between day 2 and 5 is critical for reprogramming of liver cells to induce pancreatic genes (Figure 6.34A).

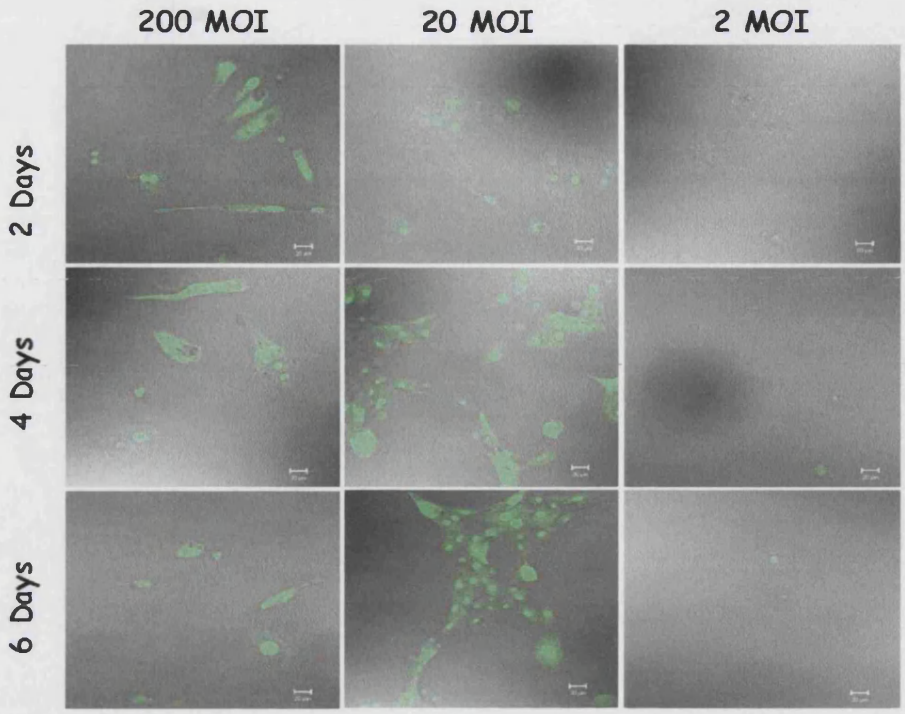


Figure 6.30 Titration of adenoviral vectors in KDS cultured rat hepatocytes. 24-hours KDS cultured rat hepatocytes were infected with different titres of Ad CMV-EGFP. The GFP expression is detected at the time points indicated. Scale bar = 20 μm.

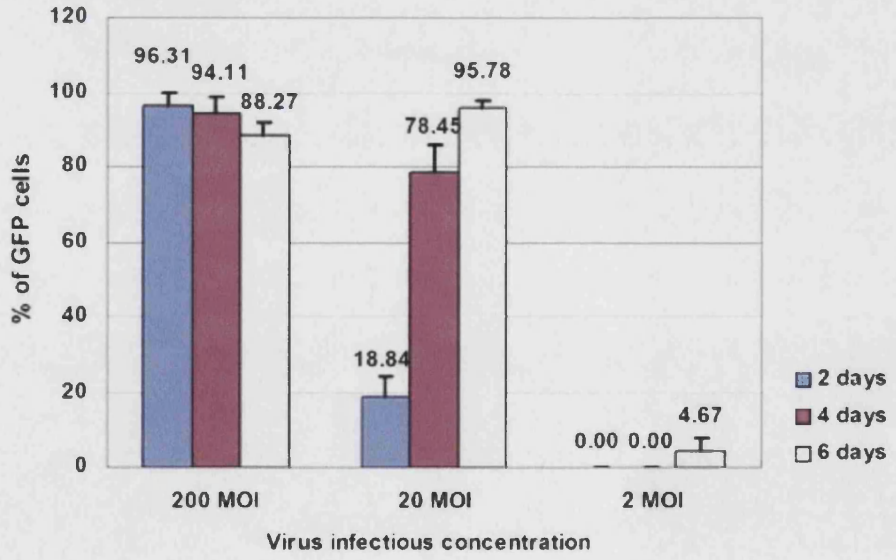


Figure 6.31 Quantitative examination of GFP positive cells in Ad CMV-EGFP infected KDS cultured rat hepatocytes. The procedure is the same as described in Figure 6.30. The percentage of GFP positive cells was determined from 6 random fields of infected cultures. The data are represented with mean + S.D.

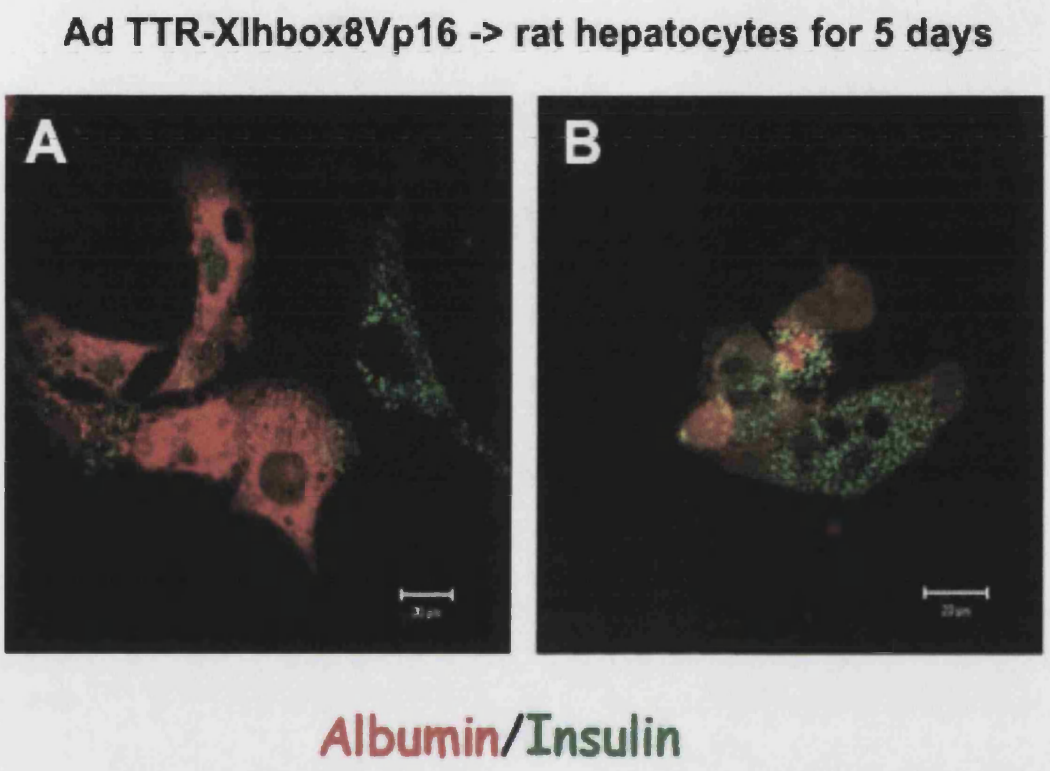


Figure 6.32 Insulin expression in cultured rat hepatocytes. Rat hepatocytes were cultured with KDS for 3 days and subsequently infected with 20 MOI Ad TTR-Xlhbox8Vp16. After a further 5-days of culture in KDS medium, the expression of insulin (green) and albumin (red) were analysed using immunoflourescence staining. Insulin is found expressed in cells either without (A) or with (B) albumin. Scale bar = 20 μm.

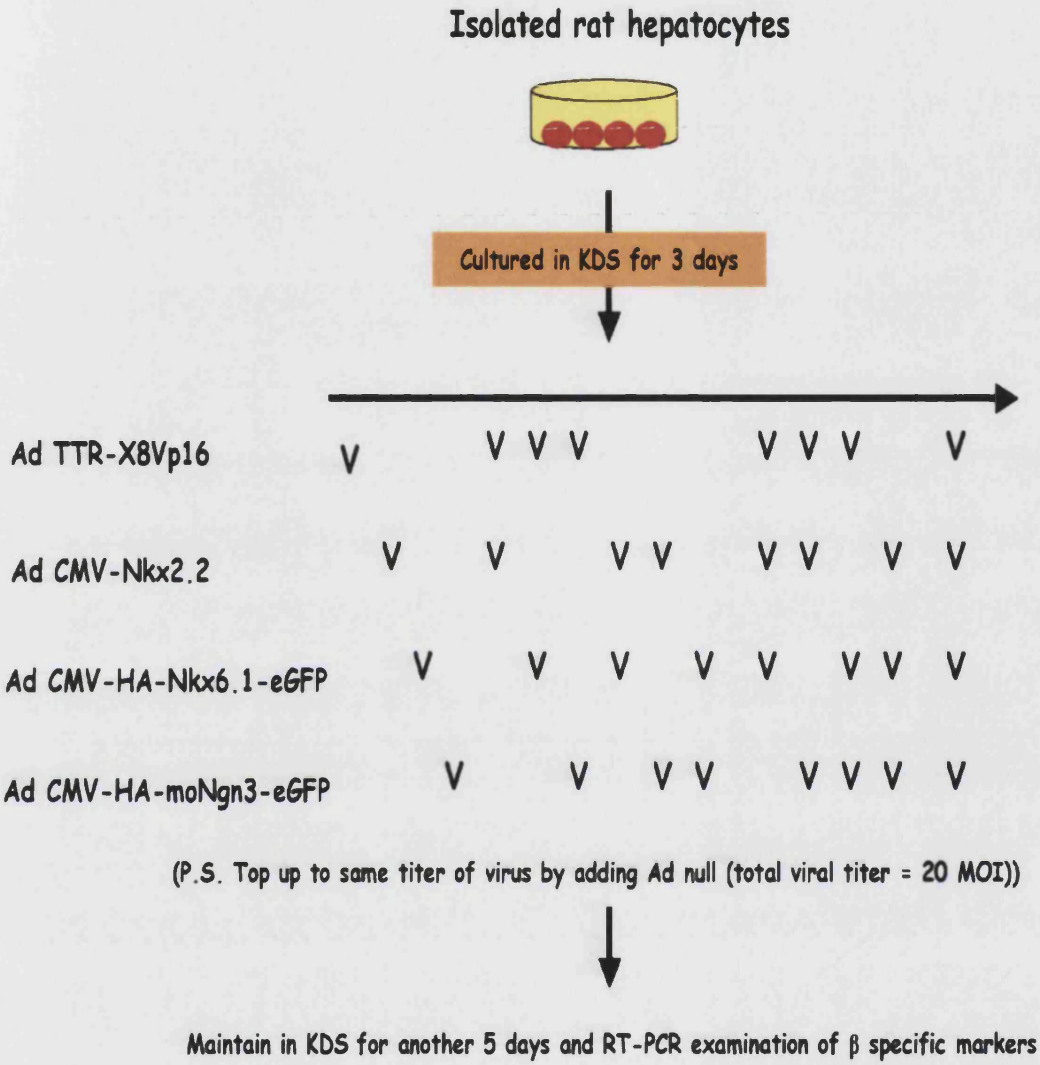


Figure 6.33 Schematic representation of the procedure to investigate pancreatic β activity in KDS cultured rat hepatocytes infected with selective combinations of adenoviruses. The adenoviruses encode pancreatic specific transcription factors.

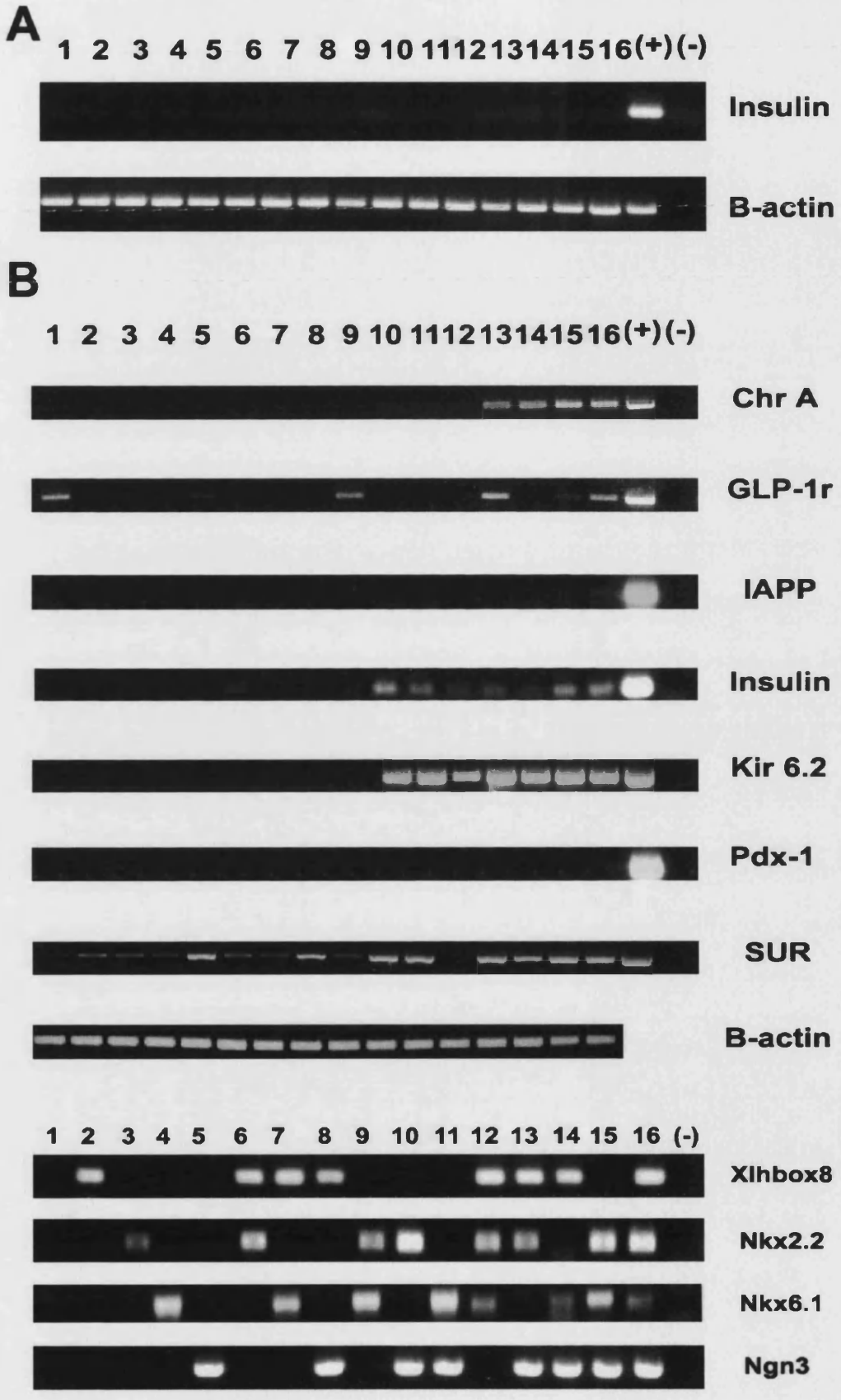

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 (+) (-)
Insulin
B-actin
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 (+) (-)
Chr A
GLP-1r
IAPP
Insulin
Kir 6.2
Pdx-1
SUR
B-actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 (-)
Xlhbox8
Nkx2.2
Nkx6.1
Ngn3

Figure 6.34 RT-PCR analysis of pancreatic β specific mRNAs in transdifferentiated pancreatic-like cells induced by the infection of various adenoviral vectors encoded pancreatic transcription factors after (A) 2 days and (B) 5 days after infection. The transcription factors were detected using RT-PCR referring to the infection combinations. Chr A, chromogranin A; GLP-1r, glucagon-like peptide 1 receptor; IAPP, islet amyloid polyeptide; Kir6.2, potassium channel subunit; SUR, sulphonylurea receptor. Neurogenin3 (Ngn3), Nkx2.2 and Nkx6.1 are all from mouse origin. (+): mRNA from rat insulinoma RIN-m5F cells; (-): no template control. Sample 1-16 are referred to the cells infected with different combinations of viruses encoding various transcription factors.

6.D Discussion

Much effort has been devoted to developing conditions suitable for *in vitro* culture of rodent or human hepatocytes. Although numerous culture media have previously been presented, it has been very difficult to find one for sustaining the differentiated state of hepatocytes (Block et al., 1996; Runge et al., 2000). Only short-term cultures has been accomplished by most groups (i.e. less than 7 days). The present work showed for the first time that KSFM medium with dexamethasone, rhEGF and BPE is able to maintain the differentiated properties of hepatocytes for up to 28 days. This statement is based on the expression of liver specific proteins (including liver-enriched transcription factors), the ability to induce drug metabolising enzymes, store lipid and produce urea.

The main benefit of KSFM culture media is that it is relatively simple in comparison with the culture conditions reported from other groups. In previous studies, supplements were added into culture medium in hepatocyte culture for different experimental purposes. They contained growth factors (EGF and hepatocyte growth factor) (Miyazaki et al., 1998), differentiation-promoting chemicals (nicotinamide and DMSO) (Mitaka, 1998), metal ions (ferrous iron, copper, manganese or zinc) (Kojima et al., 1996), and hormones/co-factors (insulin-transferrin-selenium mixture, dexamethasone and ethanolamine) (Sasaki et al., 1997; Shelly et al., 1989). Nevertheless, it is difficult to ascertain the concentration of these additives in the medium due to the different descriptions from individual groups. In contrast, commercially available supplements and dexamethasone are the only two components required to supplement the KSFM culture medium. It is also worth noting that hepatocytes were directly plated out on the culture dishes or glass coverslips without any extracellular matrix support materials such as rat tail collagen (Rana et al.,

1994), collagen/matrigel sandwich configuration (Kang et al., 2004), bone marrow stromal cells (Mizuguchi et al., 2001) or co-culture with feeder fibroblasts cells (Washizu et al., 2001). This simplifies the preparation procedure and supplies a stable system for future use.

Another interesting observation from this investigation is the potential mitogenic effect of BPE on culture rat hepatocytes. Among the supplements used in the KSFM medium, EGF has been found to induce DNA synthesis in cultured hepatocytes (Richman et al., 1976). In contrast, BPE is rarely mentioned to be essential for hepatocyte growth. In our system BPE did have an ability to support cell growth and differentiation but further investigations remain to be performed to determine the function of each component of the BPE.

Liver-enriched transcription factors such as HNF1α, HNF4α and C/EBPα are important in the control of hepatic differentiation (Costa et al., 2003). In addition, C/EBPβ has been shown to be a master regulator for the formation of transdifferentiated hepatocytes from pancreatic-derived cells (Kurash et al., 2004; Shen et al., 2000; Tosh et al., 2002b). When I examined the expression of the liver enriched transcription factors I found that HNF4α, C/EBPβ and C/EBPα expression were reduced within 3 days of culture with WE medium. This observation suggests that loss of HNF4α, C/EBPβ and C/EBPα transcription factors is the immediate cause of the loss of differentiated hepatic phenotype.

Recently, it was suggested that loss of expression of connexin proteins might be a prognostic marker for hepatocellular carcinoma (Sheen et al., 2004). The authors stated that the presence of connexin proteins in liver is associated with normal liver function. The induction of Cx32 and Cx26 proteins in DMSO-containing primary mouse hepatocyte cultures was also observed and confirmed the importance of connexin proteins in differentiating hepatoctyes (Stoehr and Isom, 2003).

A partial pancreatic phenotype can be induced following overexpression of β-cell transcription factors in cultured hepatocytes (Figure 6.34). The partial transdifferentiation of hepatocytes to pancreatic cells is also shown in recently published results from elsewhere. Kaneto et al has shown that Pdx1Vp16 in combination with NeuroD or Ngn3, enhances insulin biosynthesis (by the detection of insulin mRNA expression and the activity of insulin promoter) in liver-derived transdifferentiated cells (Kaneto et al., 2005). The data also demonstrated the enhanced expression of various pancreas-related factors including islet specific glucokinase, SUR1 and Kir6.2 in the PdX1Vp16/NeuroD or Pdx1Vp16/Ngn3 co-expressing cells. Interestingly, the gene expression of other pancreatic markers for exocrine (elastase) and endocrine (glucagon, somatostatin and pancreatic polypeptide) cells is also increased (Kaneto et al., 2005). Using the soluble factors (SFs; EGF and nicotinamide) to promote the transdifferentiation efficiency, Sapir et al generated functional pancreatic cells from isolated fetal human liver cells containing Pdx1 transgene (Sapir et al., 2005). The results indicated that the expression of pancreatic genes including endogenous human Pdx1 was 10-10^4 times greater in the presence of EGF and nicotinamide in contrast to those without treating EGF and nicotinamide. Furthermore, these transdifferentiated cells were also able to ameliorate hyperglycemia following implantation into non-obese diabetic SCID mice. In addition to EGF and nicotinamide, the effect of other soluble factors including activin A, betacellulin, Exendin-4 and Hepatocyte growth factors (HGFs) has recently been shown to be able to promote the pancreatic differentiation states of human liver-derived insulin-producing cells (Zalzman et al., 2005).

In summary, it has been described the development of a simple, novel culture system for rat hepatocytes and show that the differentiated phenotype as well as several hepatic biochemical activities can be maintained over extended periods of

culture (up to 28 days). This finding may provide a standard system for the *in vitro* culture of liver cells and could pave the way for bioengineering applications to make an artificial liver system. In addition, it is also encouraging to note that several pancreatic β cell markers can be induced when the hepatoctyes were infected with adenoviruses containing pancreatic transcription factors. This observation not only provides a powerful tool to investigate the transdifferentiation between liver and pancreas but also reveals the possibility to directly examine the exact position of individual transcription factors in the pancreatic regulatory network. Lastly, once the standard protocol to generate surrogate pancreatic β cells is established, it might be possible to test their therapeutic potential for curing diabetes.

Chapter 7. Discussion & future directions

7.A Summary

From the results presented in previous chapters, it was shown that Xlhbox8Vp16 is able to transdifferentiate two different types of liver cells (the human hepatoma cell line HepG2 or primary cultures of adult rat hepatocytes) into pancreatic cells (Chapters 3 and 6). The transdifferentiated cells express many pancreatic markers and functional properties, including glucose-sensitive insulin secretion (Chapters 3, 4 and 6). In addition, a novel culture system, using the defined media KSFM, for maintaining the differentiated properties of hepatocytes was discovered and it was shown that KSFM is able to maintain the hepatic differentiation properties for up to 28 days (Chapter 6).

7.B Is de-differentiation a prerequisite for transdifferentiation?

The ability to convert one cell type to another has enormous therapeutic potential for the treatment and cure of degenerative disorders such as diabetes and Parkinson's disease(Ball and Barber, 2003; Burke and Tosh, 2005; Lindvall et al., 2004). For example, the conversion of embryonic stem cells, pancreatic ductal (or exocrine cells) or, as shown here and in several other studies, liver cells, into pancreatic cells may make it possible to carry out many more pancreatic islet grafts on patients with severe diabetes (Baeyens et al., 2005; Hussain and Theise, 2004; Lardon and Bouwens, 2005; Nakajima-Nagata et al., 2004; Ramiya et al., 2000; Serup et al., 2001; Vaca et al., 2005) Transdifferentiation may occur through a mechanism that involves de-differentiation and re-differentiation (Bonner-Weir and Weir, 2005; Bouwens, 1998; Tsonis, 2000; Weir, 2004). For example, in chick embryos, transdifferentiation of retinal pigmented epithelial cells (PECs) to lens cells

involves de-differentiation to an intermediate phenotype before re-differentiation. Intermediate cells do not express markers for PECs or iris cell(Agata et al., 1993). Furthermore, in both amphibian heart and limb regeneration, muscle de-differentiation is commonly observed (Tsonis, 2000). More recently, Means et al. suggested that pancreatic epithelial explants undergo spontaneous acinar-to-ductal metaplasia in response to EGFR signalling. The authors concluded that this change in epithelial character is associated with the appearance of the nestin-positive transitional cells (Means et al., 2005). Nestin is expressed in the mesenchymal cells of the developing mouse pancreas (Selander and Edlund, 2002) as well as in exocrine cell types (Delacour et al., 2004) and is regarded by some as a marker of pancreatic progenitors. These findings imply that de-differentiation is an essential step to transdifferentiation in some cases. Although it is possible to induce pancreatic transdifferentiation from adult rat hepatocytes maintained in KSFM, it may be possible to enhance transdifferentiation if a different medium were used. Rather than using KSFM as a culture medium (since it maintains the hepatic phenotype extremely well), an alternative medium should be considered (e.g. one that does not sustain hepatic differentiation (e.g. Williams' Medium E).

7.C Enrichment of transdifferentiated pancreatic cells

Although some transdifferentiated pancreatic cells from HepG2 cells showed expression of the mitotic marker, phosphohistone H3, the incidence of transdifferentiated cells was still low. The reason for the low rate of transdifferentiated pancreatic cells is not known. One idea might be because the transdifferentiated cells are damaged following enforced expression of the transgene. However, dead cells

were extremely rare after transfection of TTR-Xlhbox8Vp16 into HepG2 cells (Figure 7.1). There might be another reason – the cells are damaged because the cells are forced to express the transgene at high level. This idea is supported by the observation that transgenic mice generated by crossing Alb-Cre mice with mice carrying a transgene cassette composed of the chicken beta-actin gene (CAG) promoter and a floxed linked DNA sequence (CAT) linked to *Pdx1* cDNA (Miyatsuka et al., 2003). Animals generated by this cross express pancreatic exocrine (elastase-1 and chymotrypsinogen 1B) and endocrine cell types (insulin, glucagon, somatostatin, and pancreatic polypeptide) were all found in the liver. However, the liver tissue displayed abnormal lobular structures and multiple cystic lesions. Based on the same idea, the low efficiency of transdifferentiation in the HepG2 model might be due to the catalytic activity of digestive enzymes produced by exocrine cells. To reduce the contamination of exocrine cells, endocrine specific transcription factors (such as Ngn3) rather than the general pancreatic transcription factors (such as Pdx1) might be used to direct transdifferentiation of liver cells into a restricted endocrine cell lineage. In order to isolate specific types of pancreatic cells, a reporter system could be used to sort individual cell populations. For example, to separate a pure population of pancreatic β cells two different approaches can be taken. GFP under the control of the insulin promoter can either be incorporated into the transgene or alternatively be transfected into HepG2 cells and then transdifferentiation is induced by ectopic expression of Xlhbox8Vp16. The GFP-expressing cells can be sorted by fluorescence-activated cell sorting (FACS) (Meyer et al., 1998). It has been recently shown that the sorted β cells from MIP-GFP transgenic mice (mice that express GFP under the control of the mouse insulin I gene promoter (MIP)) displayed normal β-cell properties, e.g. intracellular calcium mobilisation in response to high glucose (Hara et al., 2003). Once the cells are purified, it may be possible to culture them *in vitro* and

expand the population using known β-cell mitogens, e.g. betacellulin.

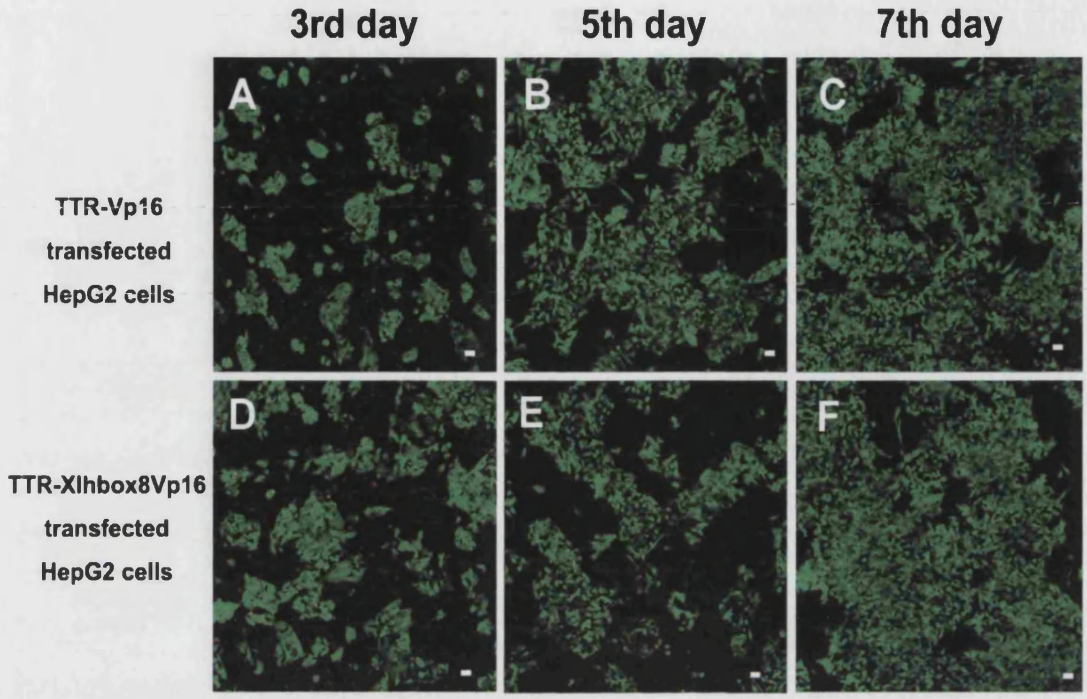


Figure 7.1 Cell viability in cultured TTR-Vp16 (A-C) and TTR-Xlhbox8vp16 (D-F) transfected HepG2 cells. The cell viability was examined using a live-dead assay in 3-day (A,D), 5-day (B, E) and 7-day (C,F) hepatocytes after transfection. Scale bar = 20 μm.

7.D High through-put screening for transdifferentiation using transgenic animals

Results from both the present investigation as well as other studies suggest Pdx1 is a master-switch gene since it can create pancreatic-like cells from non-pancreatic cells (Song et al., 1999). It will be of interest to generate transgenic animals carrying Pdx1 transgene under the control of a ubiquitous promoter (i.e. CMV promoter) to screen the possibility of conversion of non-pancreatic cell types into pancreatic cells.

In addition, once information on the role of pancreatic regulatory factors in pancreas development is elucidated the delivery of specific transcription factors at specific time points to mimic normal pancreas development (see Figure 1.1) should be carried out. It may not only be able to increase conversion efficiency but also provide more direct evidence to elucidate the role of the transcription factors in pancreas development. For instance, a transgenic mouse has recently been generated in which a pancreatic endocrine-specific enhancer drives the expression of a tamoxifen (TM)-inducible Cre recombinase/estrogen receptor fusion protein (Zhang et al., 2005). In these mice, Pdx1 can be deleted at any time point and therefore make it possible to investigate the impact of Pdx1 on pancreas development. The same strategy can also be applied to different pancreatic transcription factors (such as *Hlxb9)* whose loss of function mutation leads to pancreatic agenesis during the early pancreas development (see section 1.A.3.3).

7.E Improvements for the embryonic liver culture system

In Chapter 5, it was shown that KSFM can effectively inhibit the growth of mesenchymal cells in the embryonic liver culture system thereby making it possible to introduce the gene of interest into embryonic liver tissues. However, the poor growth of hepatic epithelium resulting from the lack of mesenchymal signals did lead to increasing cell death (Figure 5.15). To obtain the optimal condition between gene delivery efficiency and epithelial cell viability, using KSFM medium containing growth factors (see section 1.A.2) is shown to be able to rescue the epithelium phenotype without affecting the gene delivery efficiency. Nevertheless, a recent study from Serls et al indicated that response thresholds to FGFs emanating from the cardiac

mesoderm are involved in patterning the adjacent foregut endoderm into either liver or lung. This idea was supported by the observation that the hepatic protein albumin was induced when ventral foregut endoderm explants were exposed to low concentration (5 ng/ml) of Fgf1 and Fgf2 but, in contrast, the early lung-specific protein Nkx2.1 was activated when treated with high-concentration (greater than 50 ng/ml) of Fgf1 and Fgf2 (Serls et al., 2005). Furthermore, the temporal expression of Fgf receptor 4 present within the endoderm implies that signaling through Fgfr4 may be involved in specifying lung versus liver (Serls et al., 2005). This notion suggested the necessity to examine the concentration and time point when adding the growth factors into the cultured embryonic liver cells in order to maintain the hepatic epithelial phenotype.

7.F Can KSFM maintain liver differentiation in hepatocytes from different species?

Xenobiotic-induced enzyme alteration varies in hepatocytes derived from different animals (e.g. human vs rat) as well as differences within species (strain differences, human polymorphisms) (Cottrell et al., 1996). It is therefore important to examine whether the effect of KSFM is species specific or whether it can be used to maintain differentiation in hepatocytes of different origins. One encouraging preliminary result is an observation using mouse hepatocytes. Mouse hepatocytes were isolated by the same protocol as described for rat hepatocytes (collagenase perfusion procedure, described in section 2.B.2.3). The expression of albumin was detected after 72hrs based on immunostaining. In comparison, in hepatocytes cultured in Williams' Medium E, albumin expression is lost by 72 hrs (Figure 7.2). Further investigations are needed to examine both the maintenance of other hepatic markers as well as the liver functional properties of KSFM cultured mouse

hepatocytes.

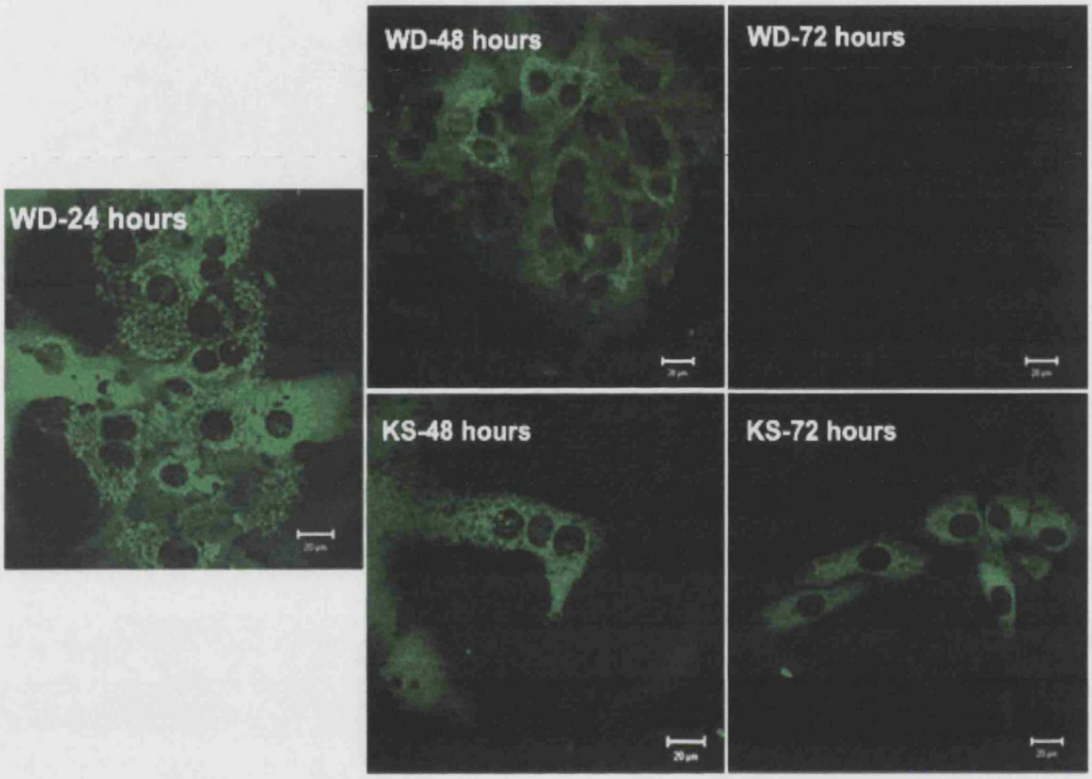


Figure 7.2 Albumin expression in adult mouse hepatocytes cultured in different culture medium for 72 hours. Albumin expression is maintained in hepatocytes cultured in KSFM medium. Scale bar = 20 μm. For abbreviations, see section 2.B.2.3.

Appendix 1 Bacterial strain / Plasmids

1.A Bacteria strains

Bacteria strains	Genotype	Supplier
E.Coli XL-1 Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB laclqZΔM15 Tn10 (Tetr)]	New England Biolabs, UK
E-Coli JM109	F`traD36 laclq Δ(lacZ)M15 pro A+ B+ / e14- (McrA -) Δ(lac-proAB)thi gyrA96 (Nalr) endA1 hsdR17(rk- mk+) relA1 supE44 recA1	Promega, UK
E-Coli DH-5 α	F- φ 80dlacZ Δ M15 Δ (lacZYA-argF)U169 deoR, recA1 endA1 hsdR17(rk- mk+ phoA supE44 λ - thi-1 gyrA96 relA1	Promega, uk

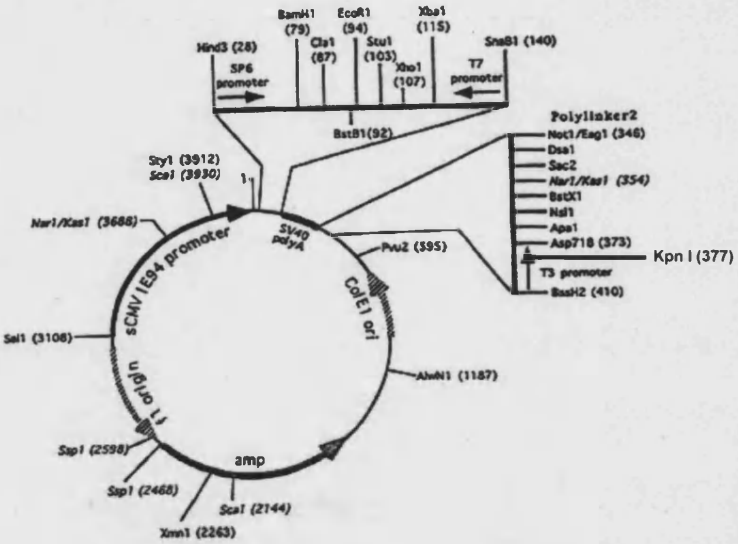
1.B Reagents for the preparation of competent cells and plasmids

Reagents	Composition	Supplier
Luria-Bertani (LB) broth	Dissovled 1 tablet in 50 ml distilled water	Sigma, UK
LB agar	Dissovled 1 tablet in 50 ml distilled water	
Ampicillin	50mg/ml stock, store in -20° C	
Restriction enzymes + buffers	different enzymes are working in specific buffer conditions	New England Biolabs, UK
T4 ligase	Stock= 400000u/ml	New England Biolabs, UK
Calf intestinal alkaline phosphatase (CIP)	Stock= 1u/ μ l	Promega, UK
DNA Polymerase I, Large (Klenow) Fragment	retain 3'→ 5' exonuclease activity lack of 5'→ 3' exonuclease activity	New England Biolabs, UK
10X DNA loading dye	10X diluted for further use	Fermentas
DNA markers (1kb DNA ladder)	1mg/ml stock, mix 50 μ g with in 50 μ l 10X DNA loading dye in 400 μ l TE buffer	Gibco^{TM}/Invitrogen Life Technologies
Agarose (Electrophoresis grade)	Powder	Gibco^{TM}/Invitrogen Life Technologies
Ethidium Bromide	Stock=0.5mg/ml	Sigma, UK

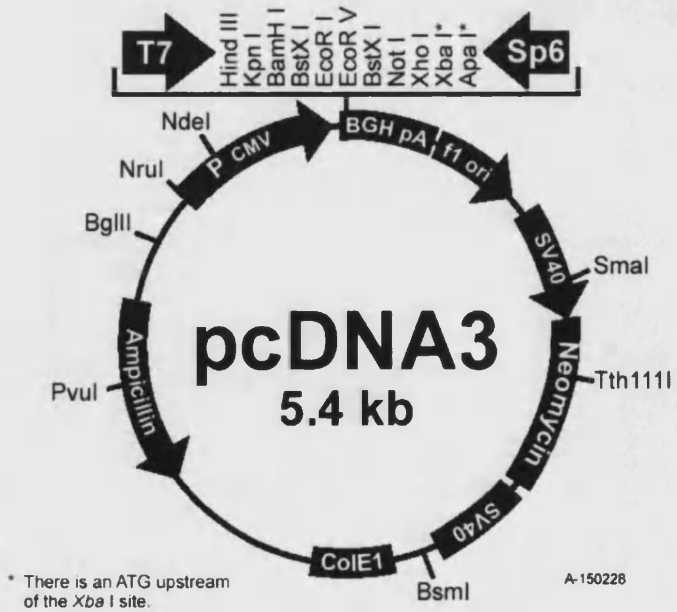
1.C Plasmid constructs

The DNA vectors used in this project were mainly constructed in 2 expression vector: pCS2+ and pcDNA3. Before the description of the experimental constructs, these 2 backbones were presented first.

A. pCS2+ (first obtained from Dr. Dave Turner, University of Michigan)

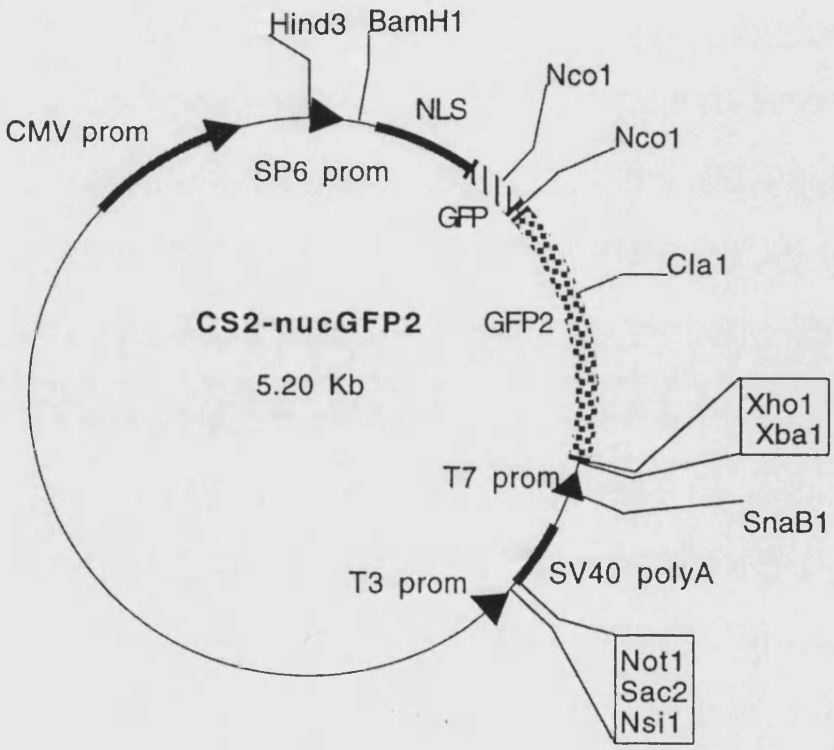


B. pcDNA3 (obtained from Gibco^{TM}/Invitrogen Life Technologies)



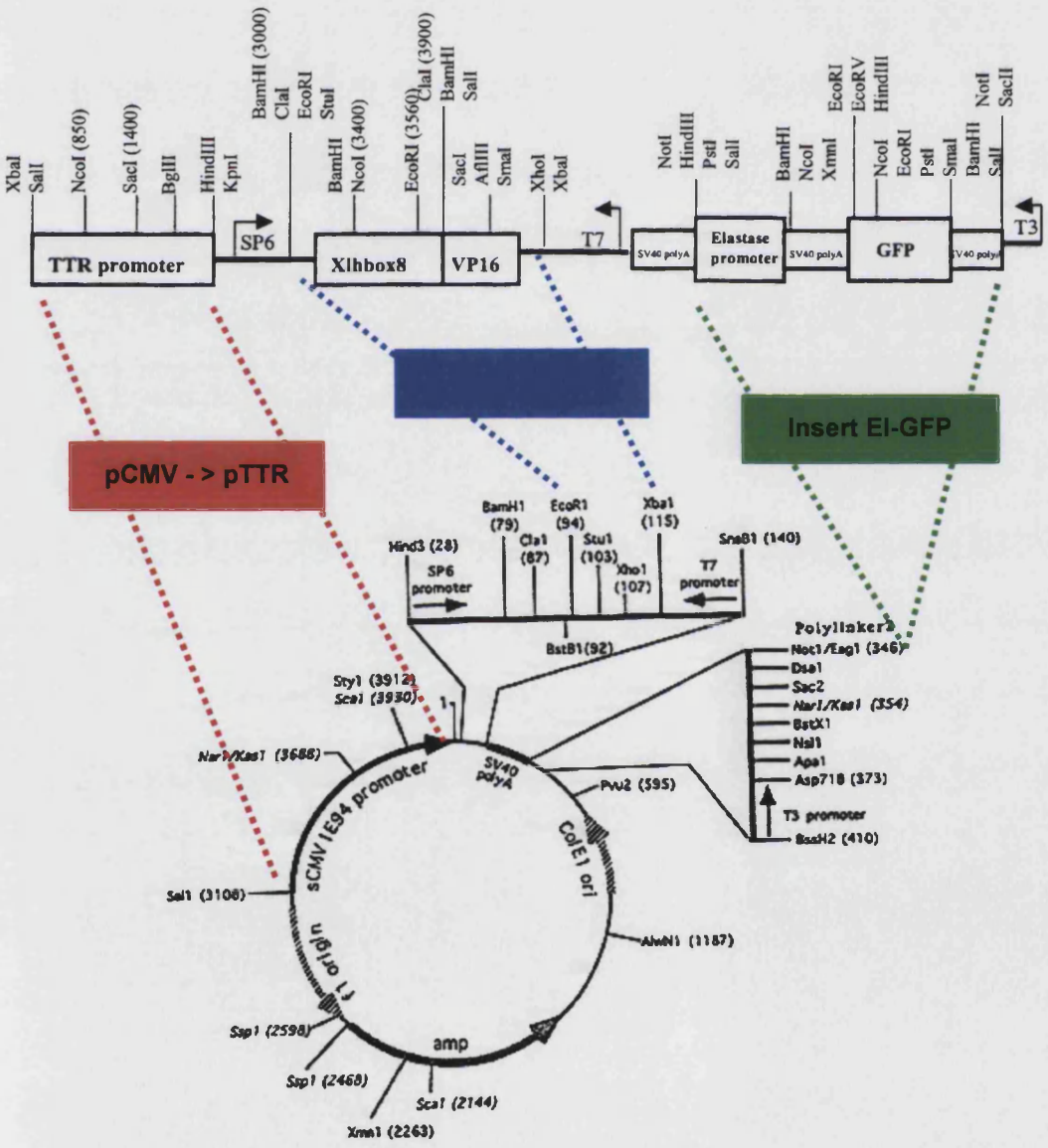
1. pCS2-nGFP

The construct was generated by Dr. Besty Pownall.



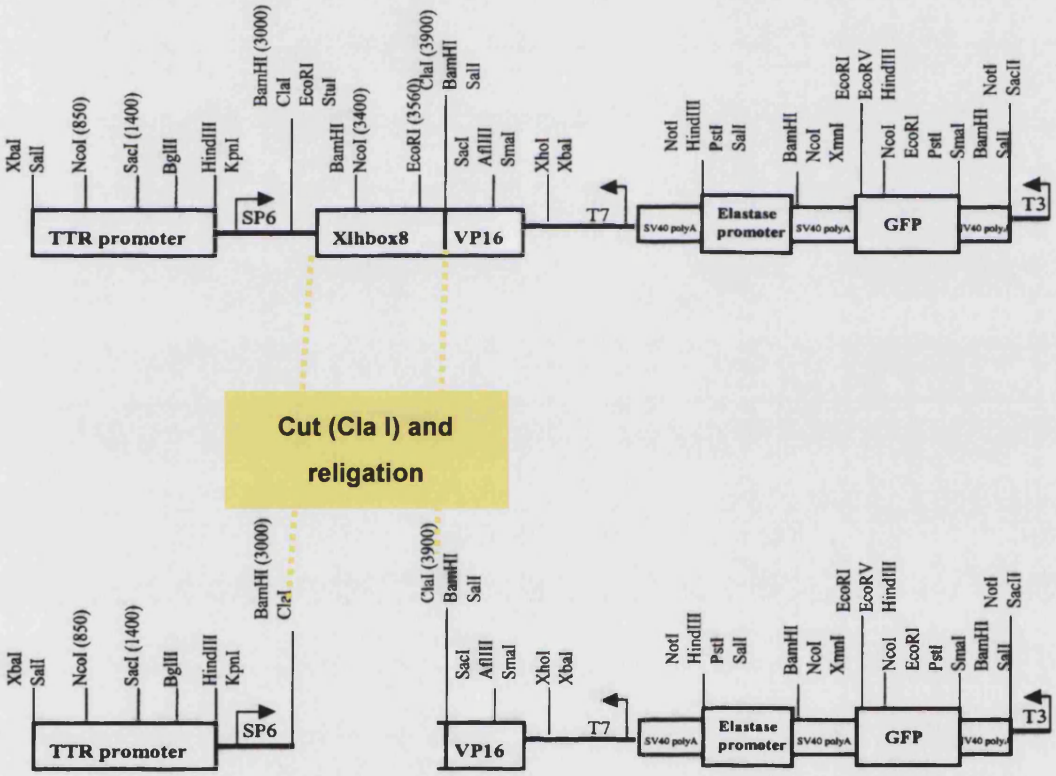
2. pCS2-TTR-Xlhbox8Vp16; Elastase-GFP

This construct was made by Dr. Marko E.Horb and all the details was described in the Experimental Procedures section in (Horb et al., 2003). The map is shown as followed.



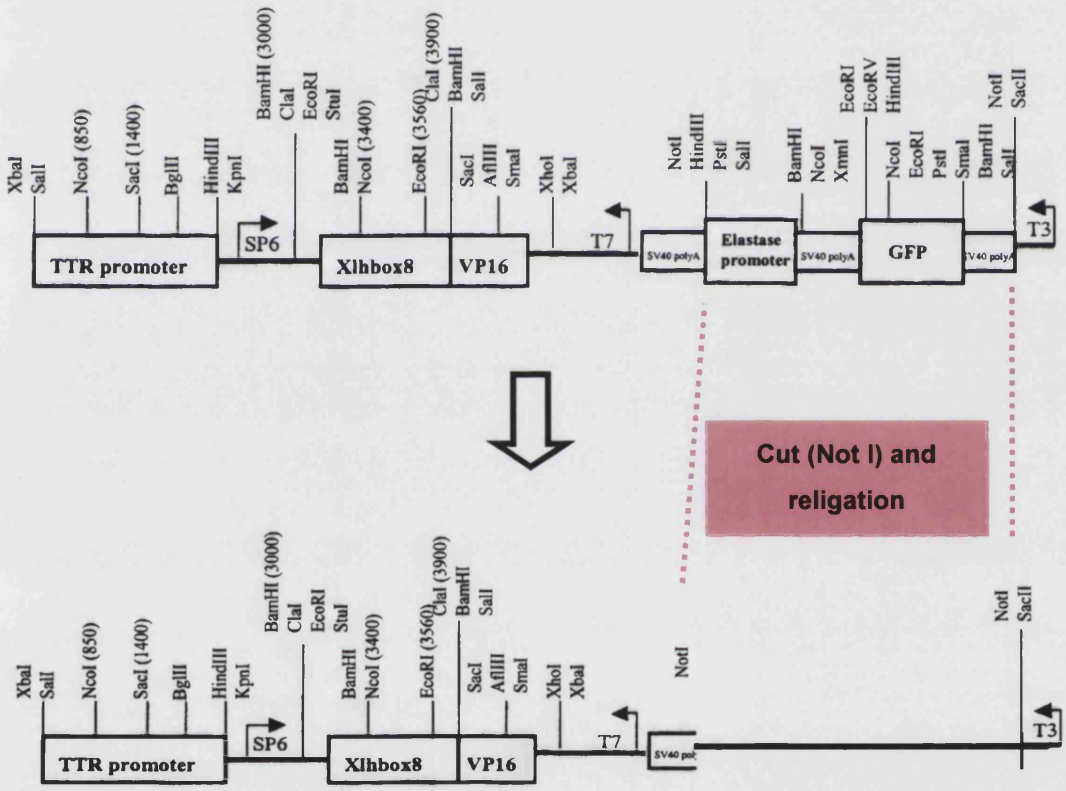
3. pCS2-TTR-Vp16; Elastase-GFP

This construct was made by Dr. Marko E.Horb for the control transfection experiment. The Xlhbox8 region was removed by cutting with Cla I and relinearised using T4 ligase. The map is shown as foilowed.



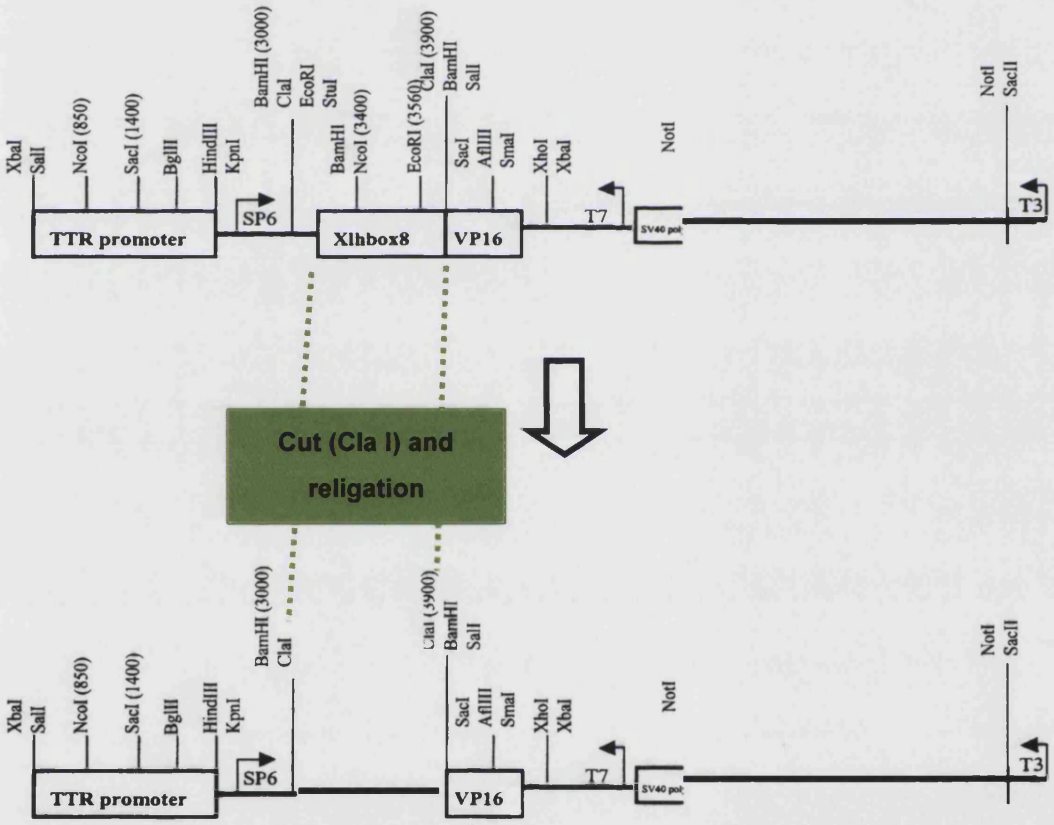
4. pCS2-TTR-Xlhbox8Vp16

The construct was constructed by removing *Elastase-GFP* portion in *pCS2-TTR-Xlhbox8Vp16; Elastase-GFP* with Not I enzyme and relegating using T4 ligase. The map is shown as followed.



5. pCS2-TTR-Vp16

This control construct was made by removing Xlhbox8 part in *pCS2-TTR-Xlhbox8Vp16* with Not I enzyme and relegating using T4 ligase. The map is shown as followed.



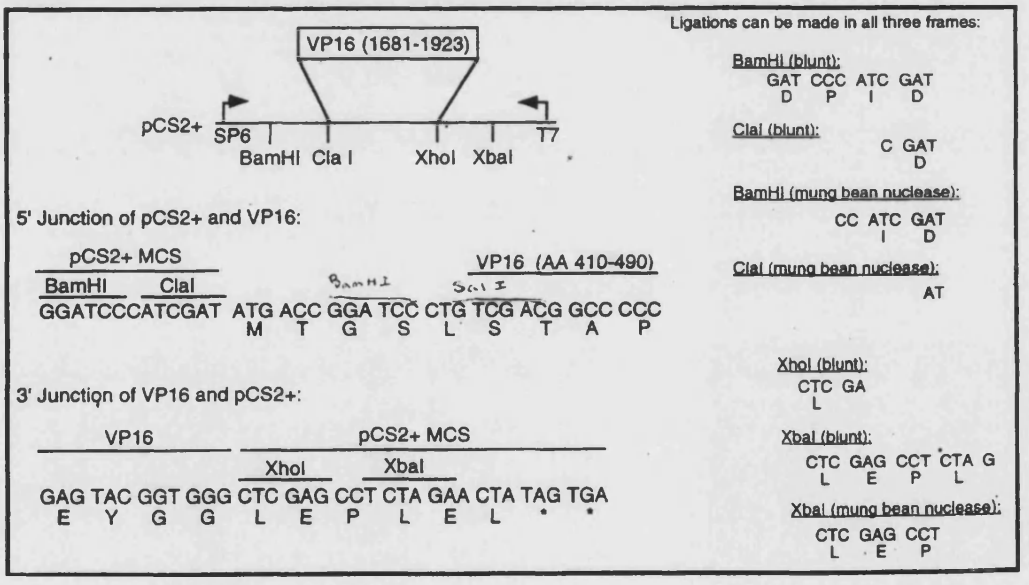
6. pCS2-Vp16-N

This construct was provided by Dr. DS Kesseler (Department of cell and Developmental Biology, University of Pennsylvania, USA). The details of the vector was shown below.

Plasmid Name VP16-N
Other Names
Constructed by Dan Kessler
Vector (name and size) pCS2+ (4095 b.p.)
Insert (name and size) VP16 activator domain (255 b.p.)
Host Strain
Selection Ampicilin
Purpose and Notes
Designed for the fusion of DNA-binding domains to the C-terminus. N-terminal fusions can also be constructed using the BamHI site in the pCS2+ polylinker. Injection into embryos has no effect.

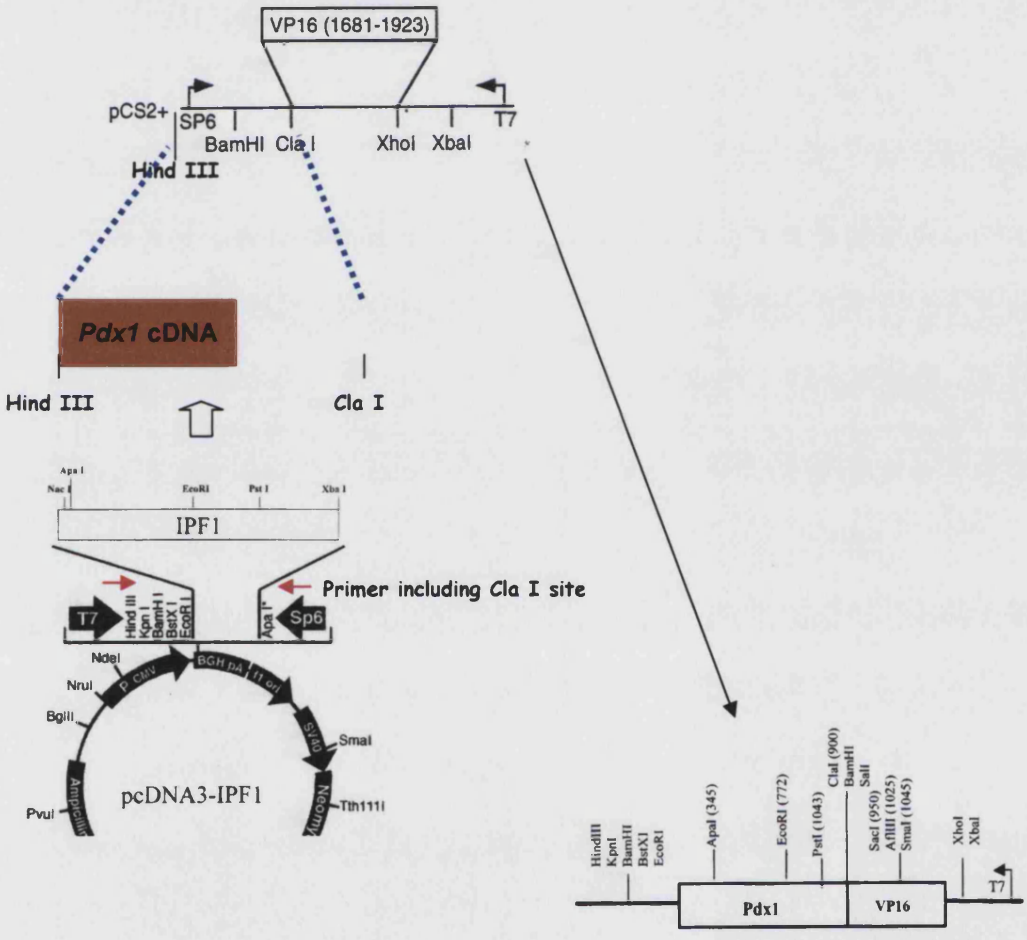
TRANSCRIPTION
For sense transcripts :
Cut with Sac II
Transcribe with SP6
For antisense transcripts:
Cut with
Transcribe with

Map **Reference** DS Kessler unpublished, 1996



7. pCS2-Pdx1Vp16

The construct was composed by Dr. Marko E Horb. It was made by putting Pdx1 cDNA (generated by PCR using primers designed for making Pdx1 cDNA from pcDNA3-IPF1 plasmid, see below) into pCS2+-VP16-N vector digested with Hind III and Cla I.

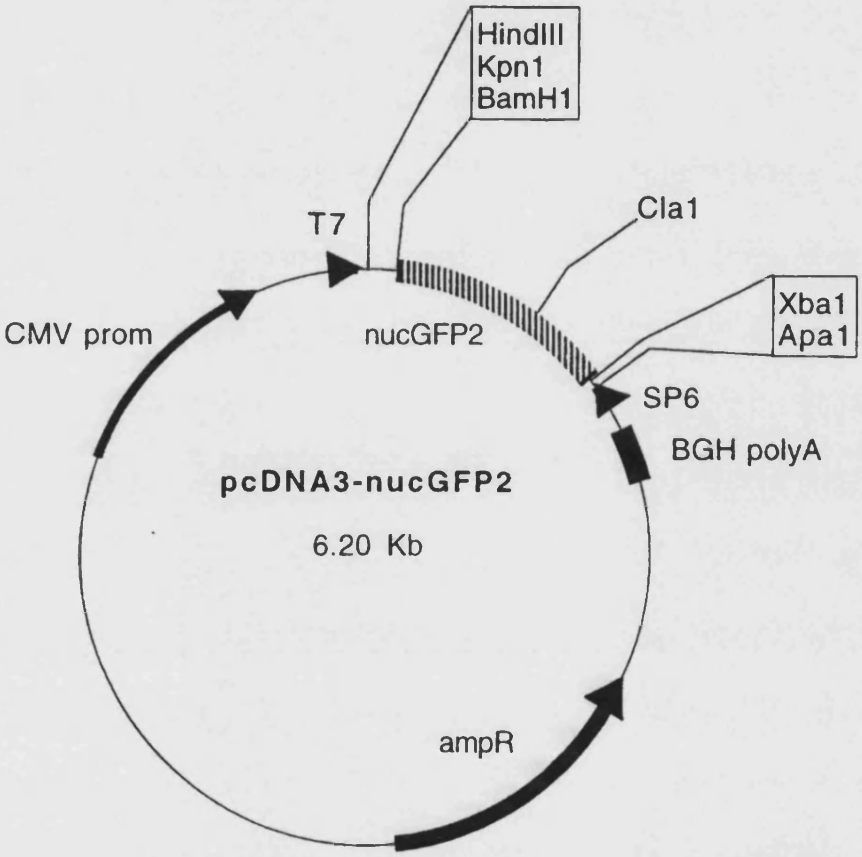


Junction of Pdx1 and VP16:

Pdx1			VP16								
			ClaI				BamHI			SalI	
CCC	TCC	AGC	ATC	GAT	ATG	ACC	GGA	TCC	CTG	TCG	ACG
P	S	S	I	D	M	T	G	S	L	S	T

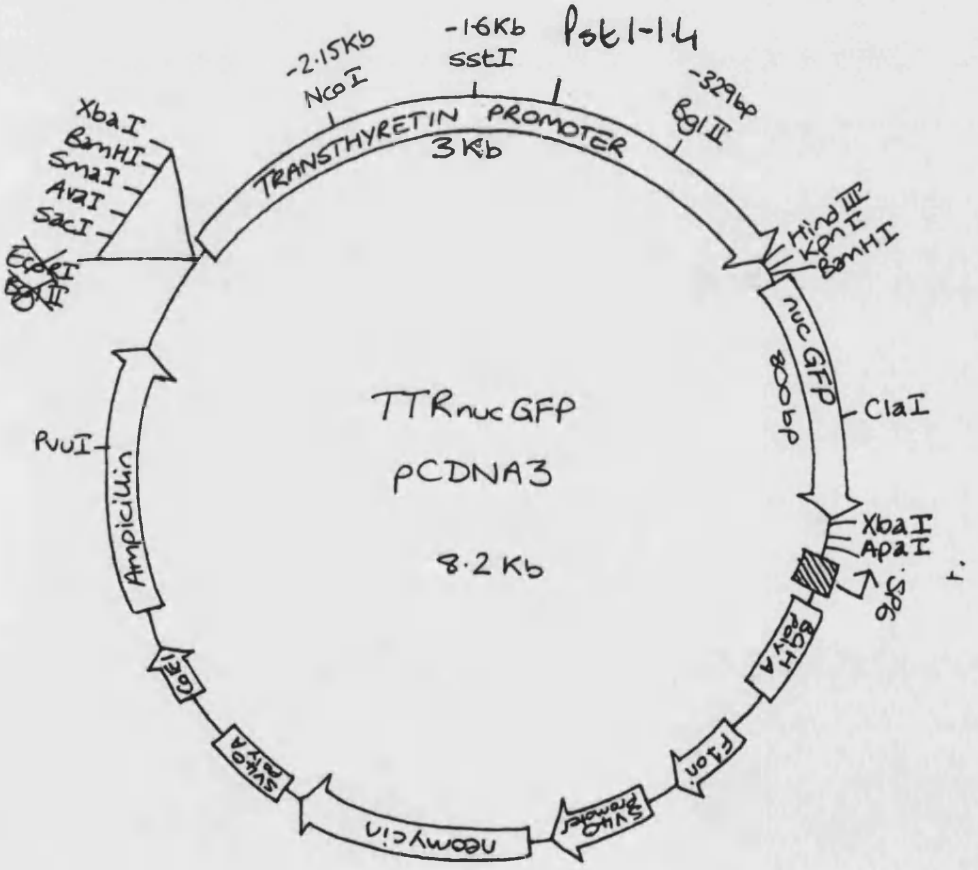
8. pcDNA3-nGFP

The construct was made by Dr. David Tosh. The nuclear GFP was cut from pCS2+-nGFP plasmid with BamHI / XbaI and cloned into BamHI / XbaI of pcDNA3 vector.



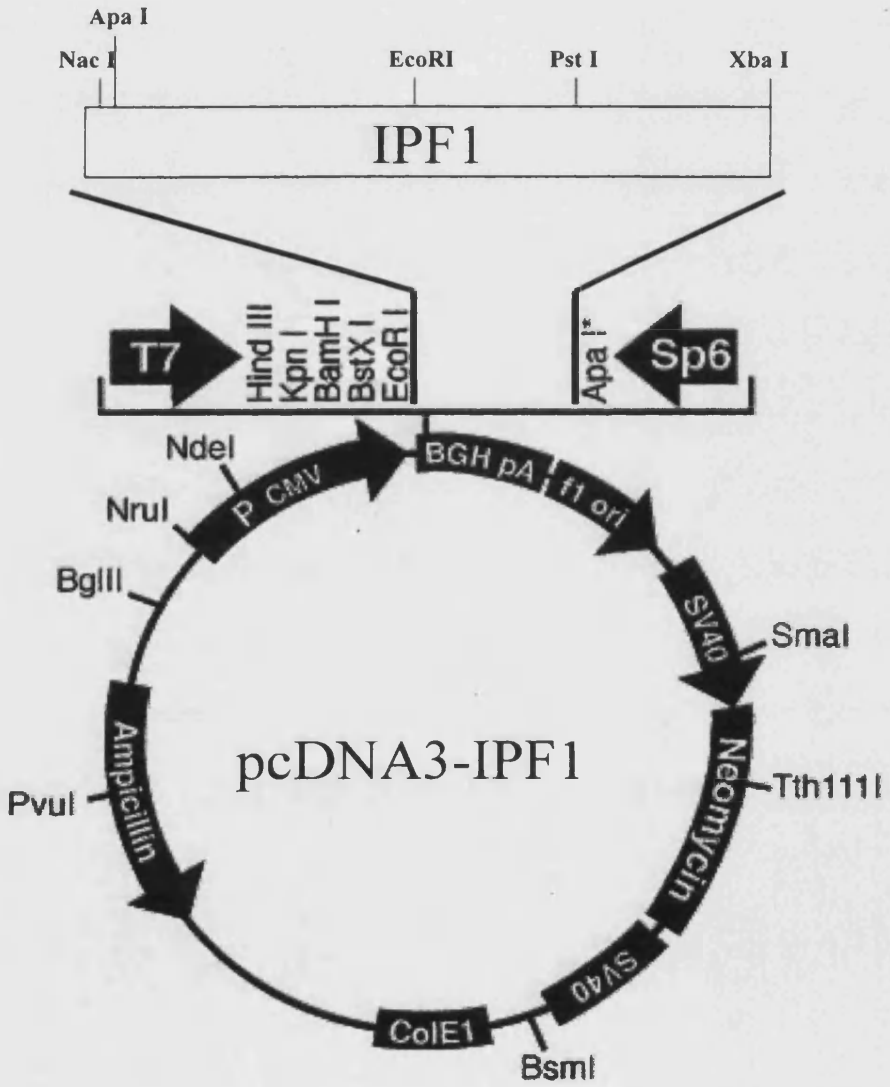
9. pcDNA3-TTR-nGFP

The plasmid was made by Dr. David Tosh. The TTR promoter (from Dr. Robert Costa, Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago) was cut with EcoRi (blunt) / Hind III and cloned into pcDNA3 excised by Bgl II (blunt) / Hind III. See (Beck and Slack, 1999) for reference.



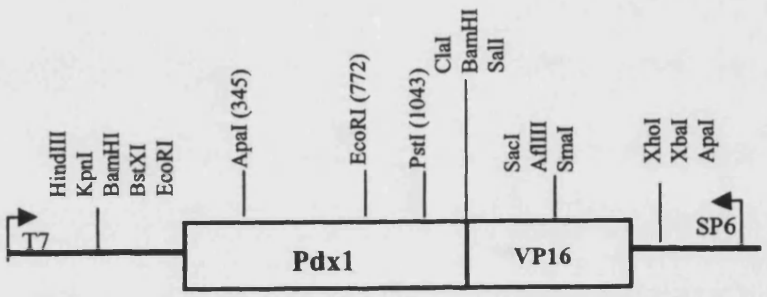
10. pcDNA3-Pdx1

The plasmid was cloned by Dr. Chia-Ning Shen. Briefly, the IPF1/Pdx1 transcript and was excised using EcoRI/XbaI from an original vector pKK4-IPF1 (kindly provided by Dr. Ulf Ahlgren) and the pcDNA3 vector was also cut with EcoRI/XbaI. The 2 fragments were then ligated with T4 ligase. The map is shown below.

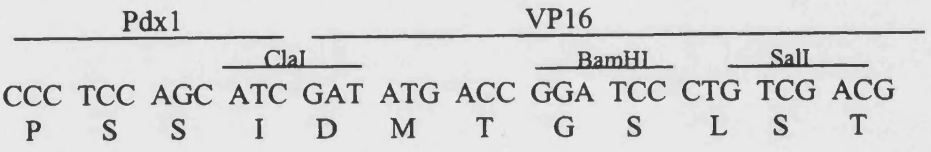


11. pcDNA3-Pdx1Vp16

The plasmid was constructed by Dr. Marko E Horb. The Pdx1Vp16 DNA was cut with Hind III/Xbal from pCS2+-Pdx1Vp16 and cloned into pcDNA3 digested with Hind III/Xbal.

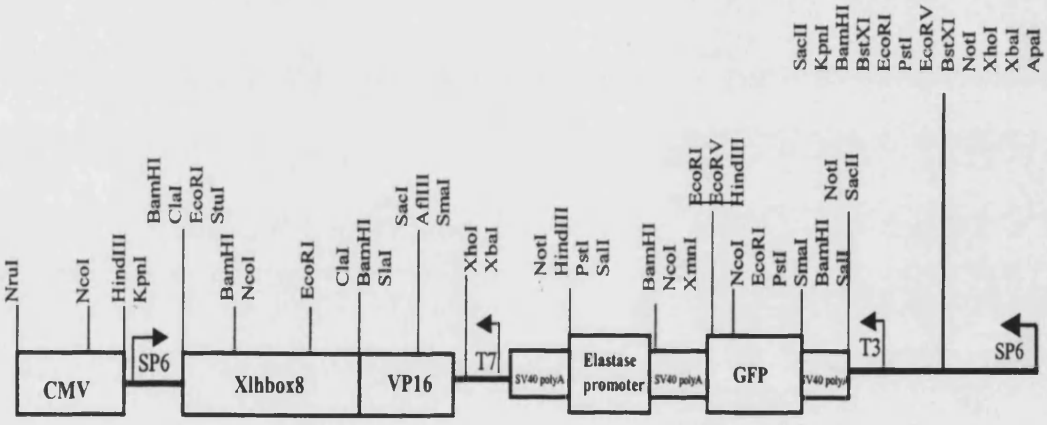


Junction of Pdx1 and VP16:



12. pcDNA3-CMV-Xlhbox8Vp16; Elastase-GFP

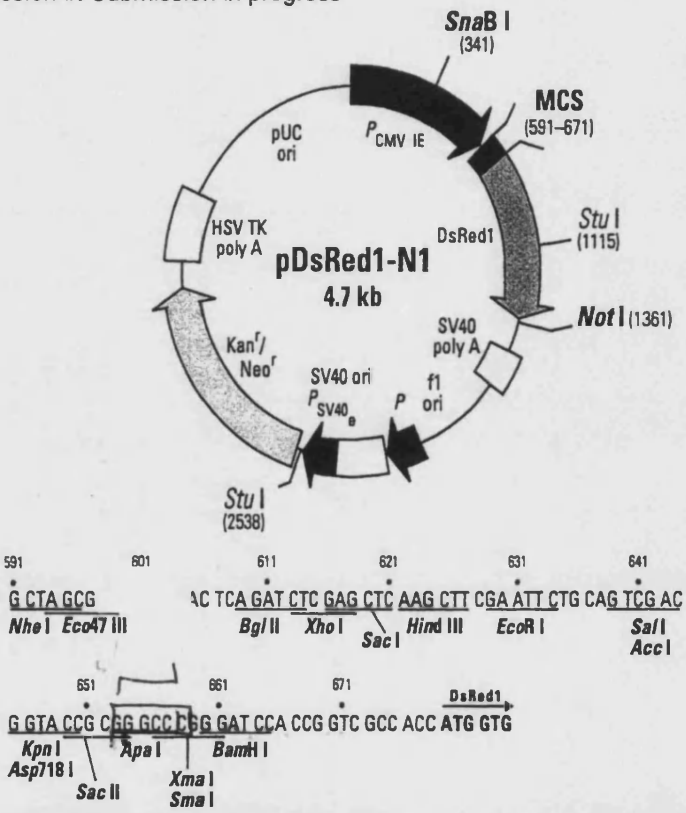
The construct was made by Dr. Marko E Horb. The Xlhbox8vp16; El-GFP fragment was cut out from *pCS2-TTR-Xlhbox8Vp16; Elastase-GFP* with Kpn I and cloned into pcDNA3 vector cut with Kpn I also.



13. pDsRed-N1 (purchased from clontech)

pDsRed1-N1 Vector Information PT3405-5

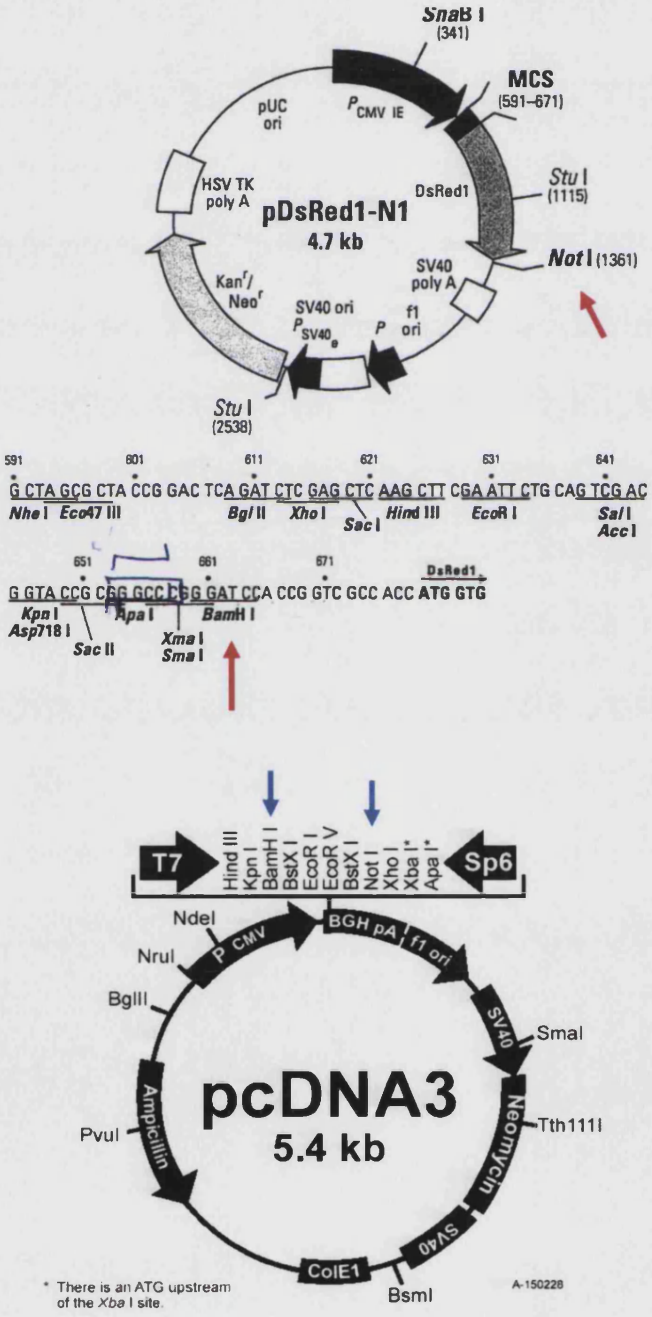
GenBank Accession #: Submission in progress Catalog #6921-1



Restriction Map and Multiple Cloning Site (MCS) of pDsRed1-N1 Vector. Unique restriction sites are in bold. The Not I site follows the DsRed1 stop codon.

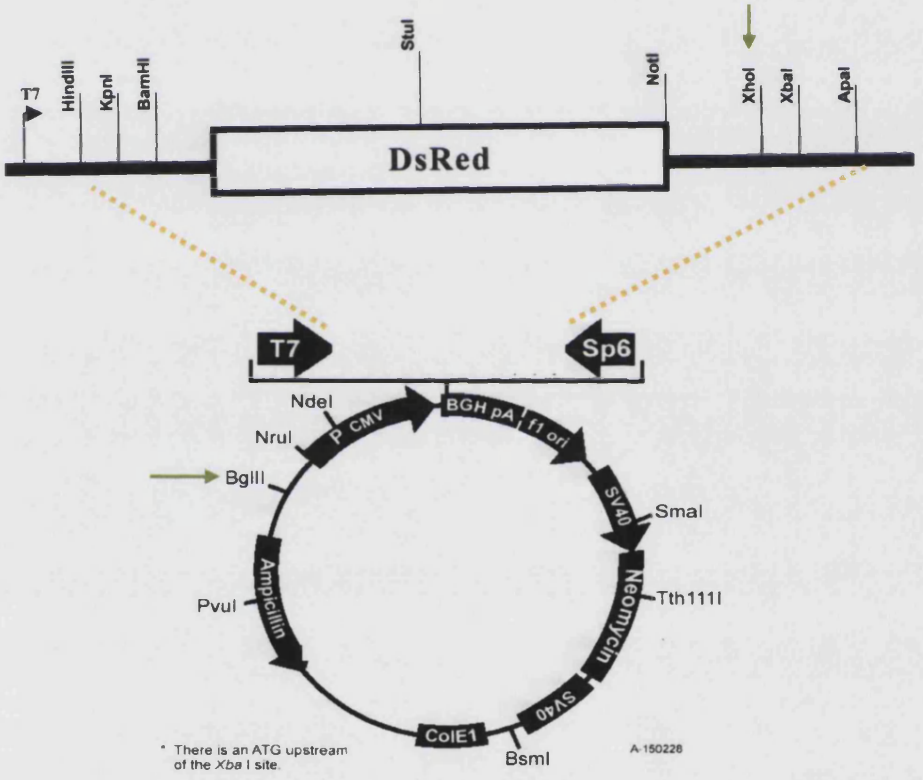
14. pcDNA3-CMV-DsRed

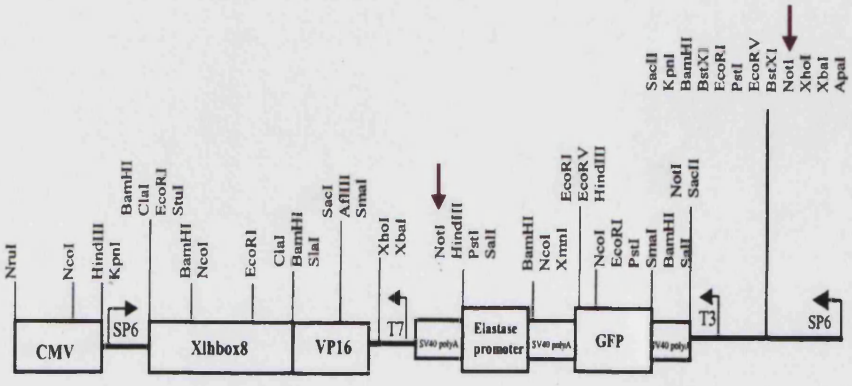
The plasmid was made by cutting pDsRed-N1 with Bam HI and Not I to excise the DsRed portion and then cloning this part into the pcDNA3 vector digested with Bam HI / Not I.



15. pcDNA3-CMV-Xlhbox8Vp16; CMV-DsRed

The plasmid was construct by replacing Elastase-GFP protion with CMV-DsRed (digested with Bgl II / Xho I then blunt by Klenow fragment) in pcDNA3-CMV-Xlhbox8Vp16; El-GFP plasmid. The pcDNA3-CMV-Xlhbox8Vp16; El-GFP was treated with Not I to remove the El-GFP part and then blunt by Klenow fragment. The 2 blunt-end DNA pieces were then ligated with T4 DNA ligase.





Appendix 2 List of Suppliers

Abcam	Cambridge, Uk
ABgene	Surrey, UK
Acris Antibodies	Middlesex, UK
Amersham Bioscience	Buckinghamshire, UK
Baxter Healthcare Ltd.	Newbury, UK
BD Bioscience	San Diego, CA, USA
BD Transduction Laboratories	San Diego, CA, USA
Beckman	Buckinghamshire, UK
Becton and Dickinson	Oxford, UK
BIOCHROM AG	Berlin, Germany
Biomeda	Foster city, CA, USA
Bio-Rad Laboratories	Herts, UK
Calbiochem	Nottingham, UK
Cambridge Biosciences	Cambridge, UK
Chemicon International	Temecula, CA, USA
Clontech	Palo Alto, CA, USA
Cypex Ltd.	Dundee, UK
DakoCytomation	Ely, UK
Ethicon Endo-Surgery	Bracknell, UK
European Collection of Cell Cultures	Salisbury, UK
Fermentas	Sunderland, UK
Fisher Scientifics	Loughbotough, UK
Fisons scientific apparatus	Loughborough, UK
Gibco™/Invitrogen Life Technologies	Paisley, UK
Gore-Tex	Newark, USA
ISTCP	New Jersey, USA
Jencons (Scientific) Ltd	Bedfordshire, UK
Leica	Milton Keynes, UK
Mercodia AB	Sweden
Millipore Corporation	Bedford, MA, USA
Molecular Probes	Leiden, The Netherlands
MP Biomedicals UK	London, UK

New England Biolabs	Hertfordshire, UK
Novagene	Madison, WI, USA
Pall Corporation	Pansacola, FL, USA
Perkin Elmer	Cambridgeshire, UK
Pierce Biotrchnologies Inc.	Rockford, IL, USA
Promega	Madison, WI, USA
Qiagen	Crawley, UK
Raymond A LAMB limited	East sussex, UK
Roche (Boehringer Mannheim)	Lewes, UK
Santa Cruz Biotechnology	Wiltshire, UK
Scientific Laboratory Supplies Ltd.	Nottingham, UK
Sigma	Poole, UK
TERUMO MEDICAL CORPORATION	Merseyside, UK
Triple Red Limited	Buckinghamshire, UK
Upstate Biotechnologies	Dundee, UK
Vector Laboratories	Burlingame, CA, USA
VWR International	Dorset, UK
Worthington Biochemical Corporation	Lakewood, NJ, USA
Zeiss	Hertfordshire, UK
Zymed Laboratories Inc.	Cambridge, UK

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